REMARKS

Entry of the foregoing, reexamination and further and favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 C.F.R. § 1.112, are respectfully requested.

By the foregoing amendment, claims 1, 4, 6, 17, 29 and 33 have been amended. In particular, claims 1 and 4 have been amended to correct typographical errors which were inadvertently inputted in the last listing of the claims. Claim 1 has been further amended to recite that "R^2 represents (a) a C_1 to C_4 lower alkyl group substituted with a COOH group, a halogen atom, a C_1 to C_4 lower alkoxy group, an amino group, a methylamino group, a dimethylamino group, a carboxymethylamino group or a carboxyethylamino group . . . ." Claims 6 and 33 have been amended in accordance with the Examiner's suggestion to insert the word "composition". Claim 17 has been amended to delete recitation of "coronary diseases including". Finally, claim 29 has been amended to delete recitation of "3-(3-amino-4-methylbenzenesulfonyl)-7-chloro-2,4(1H,3H)-quinazolinedione". Support for all of the above amendments can be found throughout the originally filed application. No new matter has been added.

Turning now to the Official Action, applicants acknowledge the Examiner's statement that claims 26 and 32 have been allowed. *See* OFFICE ACTION SUMMARY ¶ 5.

Claims 3, 14-17, 21 and 29 have been objected to as being dependent upon a rejected base claim. The Examiner has indicated that such claims "would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims." *See* OFFICE ACTION at 2, ¶ 3. For the reasons

discussed below, the rejection(s) of the base claims for which claims 3, 14-17, 21 and 29 depend should be withdrawn. As such, claims 3, 14-17, 21 and 29 should be allowable without having to be rewritten in independent form.

Claims 1-2, 4-6, 17, 20 and 22 have been rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Fukami et al. (U.S. Patent No. 5,814,631) (hereinafter "US '631"). This rejection is respectfully traversed.

In proceedings before the Patent and Trademark Office, the Examiner bears the burden of establishing a *prima facie* case of obviousness based upon the prior art. The Examiner can satisfy this burden only by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references. *See, e.g., In re Fritch*, 23 U.S.P.Q.2d 1780, 1783 (Fed. Cir. 1992).

Here, the Examiner has stated that US '631 teaches the synthesis of 3-(4-aminobenzenesulfonyl)-7-chloro-2,4-(1H,3H)-quinazolinedione, which "is almost the same" as the applicants' claimed quinazoline derivative having the formula (1). OFFICE ACTION at 2-3. The Examiner further stated that the only difference between applicants' claimed quinazoline derivative and the species allegedly disclosed in US '631 is the R^2 substitutent in formula (1). OFFICE ACTION at 3. In particular, the Examiner stated that applicants' claims require "R^2 to be methyl," but R^2 in the cited compound of Example 148 of US '631 is hydrogen. OFFICE ACTION at 3.

However, contrary to the Examiner's statement, applicants' claims do not require R^2 to be methyl. Rather, R^2 represents any one of (a) through (n) as defined in the claims. Should R^2 be (a) and the C_1 to C_4 lower alkyl group of (a) be methyl (or any other C_1 to C_4 lower alkyl group for that matter), the alkyl group is to be

"substituted with a COOH group, a halogen atom, a C_1 to C_4 lower alkoxy group, an amino group, a methylamino group, a dimethylamino group, a carboxymethylamino group or a carboxyethylamino group[.]" US '631 fails to teach or even suggest that the methyl group or any C_1 to C_4 lower alkyl group of R^2 is to be "substituted with a COOH group, a halogen atom, a C_1 to C_4 lower alkoxy group, an amino group, a methylamino group, a dimethylamino group, a carboxymethylamino group or a carboxyethylamino group[.]"

Although the Examiner argues that the change of the hydrogen substituent to a methyl group would have been obvious because US '631 already teaches a methyl substituent on the phenyl group, the change of the hydrogen substituent to the above-specified substituted alkyl (e.g., methyl) group is neither taught nor suggested by US '631.

Accordingly, a proper *prima facie* case of obviousness has not been established against applicants' claimed invention. As such, the Examiner is respectfully requested to withdraw this § 103 rejection over US '631.

Claim 17 has been rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for the treatment of most of the diseases, purportedly does not provide enablement for the treatment of coronary diseases. Applicants respectfully traverse this rejection.

To expedite prosecution in the present application and not to acquiesce to the Examiner's rejection, claim 17 has been amended such that the coronary disease now recited by claim 17 is specifically cardiac infarction. The effectiveness of the chymase inhibitors for the treatment of the cardiac infarction is described in the references attached hereto as Exhibits 1-4.

As reported in Exhibit 1 (Sadoshima et al., Circ. Res., 73(3):413-23 (1993)), it is known in the art that angiotesin II (Ang. II) may act as a growth factor for the heart. More specifically, Ang. II induces the hypertrophy of cardiac cells, differentiation of fibroblast and production of collagen from fibroblast. Further, it is considered from the report in Exhibit 2 (Balcells et al., Am. J. Physiol., 273(4Pt2):H1769-74 (1997)), *i.e.*, the production of Ang. II from chymase is predominant over that of ACE in the human cardiac texture, that the excessive production of Ang. II by chymase plays an important role for the pathology after the cardiac infarction. Exhibit 3 (Pitt et al., Lancet, 349:747-52 (1997)) reports that, although ACE inhibitor and Ang. II antagonist improve the cardiac function after the cardiac infarction, the total death rate of the Ang. II antagonist is significantly lower, and therefore, there are differences in the aftercare improvement effects. This clearly shows that Ang. II produced from chymase, which is Ang. II production enzyme other than ACE, participates the tissue remodeling after the cardiac infarction. Additionally, Exhibit 4 (Yamagishi et al., 25(11):1369-80 (1993)) reports that, in the study of rats, the contents of Ang. II in the cardiac ventricular tissues, rather than those in the circulating blood, have an effect on the increase of ventricular weight.

Thus, the disclosure of the present application together with the contemporary knowledge in the field at the time the application was filed, adequately describes the effectiveness of chymase inhibitor for the treatment of the postmyocardial infarction. Accordingly, withdrawal of the enablement rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claim 6, 17 and 33 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for allegedly failing to particularly point out and

distinctly claim the subject matter which applicants regard as the invention. In particular, the Examiner has suggested, concerning claims 6 and 33,[1] that applicants recite "chymase inhibitor composition". With regard to claim 17, the Examiner has indicated that use of the term "including" is indefinite. Applicants respectfully traverse the Examiner's rejection under 35 U.S.C. § 112, second paragraph.

To expedite prosecution in the present application and not to acquiesce to the Examiner's rejection, claim 17 has been amended to delete recitation of "coronary diseases including". Additionally, to expedite prosecution in the present application and not to acquiesce to the Examiner's rejection, claims 6 and 33 have been amended in accordance with the Examiner's suggestion to recite "chymase inhibitor composition".

In view of the above, the Examiner is respectfully requested to withdraw the rejection under 35 U.S.C. § 112, second paragraph.

From the foregoing, further and favorable action in the form of a Notice of Allowance is respectfully requested and such action is earnestly solicited.

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[1] On page 6, paragraph 5.b. of the Office Action, the Examiner stated that "[i]n claims 6 and 32, the phrase 'chymase inhibitor' should read as 'chymase inhibitor composition', since it is a composition claim. However, since the Examiner rejected claim 33, not 32, under 35 U.S.C. § 112, second paragraph and claim 33, not claim 32, includes the recited phrase, it appears that recitation of claim 32 in paragraph 5.b. was in error.

In the event that there are any questions concerning this Amendment and Reply, or the application in general, the Examiner is respectfully requested to telephone the undersigned so that prosecution of the application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

Date: October 25, 2004

By: _____
Susan M. Dadio
Registration No. 40,373

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

Molecular Characterization of Angiotensin II–Induced Hypertrophy of Cardiac Myocytes and Hyperplasia of Cardiac Fibroblasts

Critical Role of the AT_1 Receptor Subtype

Jun-ichi Sadoshima, Seigo Izumo

Increasing evidence suggests that angiotensin II (Ang II) may act as a growth factor for the heart. However, direct effects of Ang II on mammalian cardiac cells (myocytes and nonmyocytes), independent of secondary hemodynamic and neurohumoral effects, have not been well characterized. Therefore, we analyzed the molecular phenotype of cultured cardiac cells from neonatal rats in response to Ang II. In addition, we examined the effects of selective Ang II receptor subtype antagonists in mediating the biological effects of Ang II. In myocyte culture, Ang II caused an increase in protein synthesis without changing the rate of DNA synthesis. In contrast, Ang II induced increases in protein synthesis, DNA synthesis, and cell number in nonmyocyte cultures (mostly cardiac fibroblasts). The Ang II–induced hypertrophic response of myocytes and mitogenic response of fibroblasts were mediated primarily by the AT_1 receptor. Ang II caused a rapid induction of many immediate-early genes (*c-fos, c-jun, jun B, Egr-1,* and *c-myc*) in myocyte and nonmyocyte cultures. Ang II induced "late" markers for cardiac hypertrophy, skeletal α-actin and atrial natriuretic factor expression, within 6 hours in myocytes. Ang II also caused upregulation of the angiotensinogen gene and transforming growth factor-β_1 gene within 6 hours. Induction of immediate-early genes, late genes, and growth factor genes by Ang II was fully blocked by an AT_1 receptor antagonist but not by an AT_2 receptor antagonist. These results indicate that (1) Ang II causes hypertrophy of cardiac myocytes and mitogenesis of cardiac fibroblasts, (2) the phenotypic changes of cardiac cells in response to Ang II in vitro closely mimic those of growth factor response in vitro and of load-induced hypertrophy in vivo, (3) all biological effects of Ang II examined here are mediated primarily by the AT_1 receptor subtype, and (4) Ang II may initiate a positive-feedback regulation of cardiac hypertrophic response by inducing the angiotensinogen gene and transforming growth factor-β_1 gene. (*Circulation Research* 1993;73:413-423)

KEY WORDS • angiotensin II • AT_1 receptor • immediate-early genes • mitogenesis • hypertrophy

The renin-angiotensin system plays a critically important role in the control of cardiovascular and renal homeostasis.[1,2] Previously, this system has been considered to be an endocrine system, in which angiotensinogen is produced in the liver and secreted into the systemic circulation, where the successive proteolytic cleavages by renin and angiotensin converting enzyme (ACE) occur to produce the biologically active peptide angiotensin II (Ang II).[1,2]

Recently, however, there is accumulating evidence for the existence of an independent tissue (local) renin-angiotensin system in several organs. This concept is supported by evidence derived from biochemical, immunohistochemical, and molecular biological demonstration of all components of the renin-angiotensin system, including renin, angiotensinogen, ACE, angiotensin I (Ang I), Ang II, and Ang II receptors in local tissues, including the heart and blood vessels. Besides its potent vasoconstrictive effect, Ang II has been suggested to work as an autocrine/paracrine factor regulating growth of local tissues such as blood vessel, kidney, and heart.[3-7]

The direct growth effect of Ang II has been extensively characterized in vascular smooth muscle cells, where Ang II has been shown to promote hypertrophy in vitro.[8-10] Interestingly, Ang II also causes hyperplasia in some smooth muscle cells in culture, such as the aorta of spontaneously hypertensive rats or the renal arterioles of normal rats.[11,12] Thus, Ang II directly or in combination with other growth factors may play an important role in the development of vascular hypertrophy and elevated arterial resistance in hypertension.

Several studies in vivo have suggested that Ang II may also be a critical factor in mediating cardiac hypertrophy. First, chronic infusion of subpressor doses of Ang II to rats caused ventricular hypertrophy without changes in blood pressure.[13] Second, in a genetic model of hypertension, normalization of blood pressure by sympatholytic agents or by direct vasodilators did not cause regression of cardiac hypertrophy, whereas treatment with an ACE inhibitor did.[14] Third, treating the rats having abdominal aortic coarctation with an ACE

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From the Molecular Medicine and Cardiovascular Divisions, Beth Israel Hospital, and the Department of Medicine and Program in Cell and Developmental Biology, Harvard Medical School, Boston, Mass.

Correspondence to Molecular Medicine Unit, Beth Israel Hospital, Brookline Ave, Boston, MA 02215 (Dr Izumo).

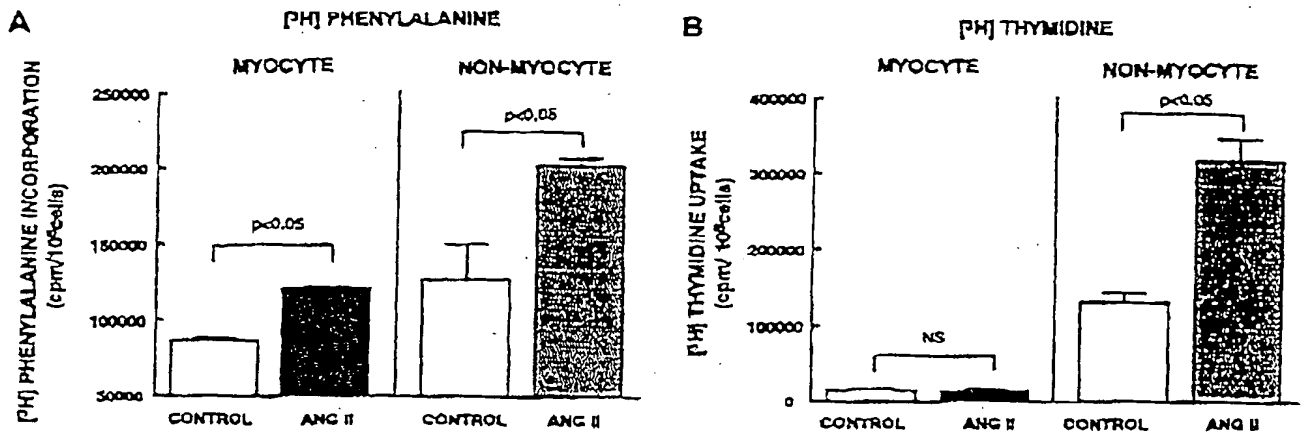

FIG 1. *Bar graphs showing the effect of angiotensin II (ANG II) on [³H]phenylalanine incorporation (A) and [³H]thymidine uptake (B) in myocytes and nonmyocytes of the neonatal rat heart. A shows [³H]phenylalanine incorporation over 48 hours; B, [³H]thymidine uptake over 24 hours. Myocyte and nonmyocyte fractions were prepared as described in the text. ANG II (10 nM) was applied every 12 hours to compensate for decrease due to degradation by an endogenous angiotensinase in culture media. Data are normalized to the mean cell counts before ANG II stimulation, which was obtained from the parallel culture dishes. Data are mean+SEM obtained from four to nine samples in each group.*

inhibitor prevented left ventricular hypertrophy, even though carotid artery pressure in these rats was not different from that in the untreated animals.[15] Fourth, treatment of newborn pigs with an ACE inhibitor interfered with the physiological hypertrophy of the left ventricle associated with normal growth.[16] Finally, treatment of patients suffering from myocardial infarction with ACE inhibitors prevented cardiac dilatation.[17] These observations are consistent with, though do not prove, the notion that Ang II may act as an endogenous growth factor for the myocardium. However, ACE inhibitors lower blood pressure and also inhibit kinin and bradykinin metabolisms.[1,2] Therefore, some effects of ACE inhibitors might not have been due to inhibition of Ang II production.

Recently, Baker et al[18] have shown that Ang II increases protein synthesis in chick cardiac myocytes in vitro. However, molecular characterization of the phenotypic changes in cardiac myocytes in response to Ang II has not been reported. Furthermore, it is not known whether Ang II has any direct effects on cardiac nonmyocyte populations (fibroblasts, endothelial cells, smooth muscle cells, etc), which account for as many as 50% of the total cell number of the heart.[19] Thus, the objectives of the present experiments are (1) to characterize the phenotypic changes induced by Ang II in cardiac myocytes and nonmyocytes (primarily fibroblasts) in vitro and (2) to examine which receptor subtype (AT_1 or AT_2) mediates biological effects of Ang II in myocytes and nonmyocytes.

Materials and Methods

Materials

All culture reagents were purchased from GIBCO, Gaithersburg, Md. All radiochemicals were obtained from Du Pont–New England Nuclear, Boston, Mass. Losartan and PD123319 were generous gifts from Du Pont Merck, Wilmington, Del, and Parke-Davis, Ann Arbor, Mich, respectively. All angiotensin-related peptides were purchased from Peninsula Laboratories, Belmont, Calif. All other chemicals were from Sigma Chemical Co, St Louis, Mo.

Preparation of Myocyte-Rich Culture

Primary cultures of the neonatal rat cardiac myocyte were prepared as described previously.[20] To selectively enrich for myocytes, dissociated cells were preplated for 1 hour, during which period the nonmyocytes attached readily to the bottom of the culture dish. The resultant suspension of myocytes was plated onto gelatin-coated 35-mm or 60-mm culture dishes at a density of 1×10^5 cells/cm^2. Bromodeoxyuridine (BrdU, 100 μM) was added during the first 24 to 36 hours to prevent proliferation of nonmyocytes. All experiments were done in the serum-free condition 24 to 48 hours after changing to the serum-free medium. Using this method, we routinely obtained myocyte-rich cultures with 90% to 95% myocytes (hereafter referred to as myocyte cultures), as assessed by microscopic observation of cell beating and by immunofluorescence staining with a monoclonal antibody (MF20) against sarcomeric myosin heavy chain.[21]

Preparation of Nonmyocyte-Rich Culture

Highly enriched cultures of cardiac nonmyocytes (hereafter referred to as nonmyocyte cultures) were prepared passing twice the cells adherent to the culture dish during the preplating procedure.[20] Until the second passage, cells were maintained in the same culture medium as above, except that 10% calf serum was used and BrdU was not used. After the second passage, the same serum-free medium as above was used. In this nonmyocyte culture, less than 10% of the cells were sarcomeric myosin positive. The percentage of myosin-positive cells did not increase with time, arguing against the notion that myosin-negative cells are undifferentiated cardioblasts. Immunostaining with an antibody against smooth muscle, α-actin (IBL Research Products, Cambridge, Mass), revealed that less than 10% of cells were positive. Incubating nonmyocyte culture with the fluorescence-labeled acetylated low density lipopro-

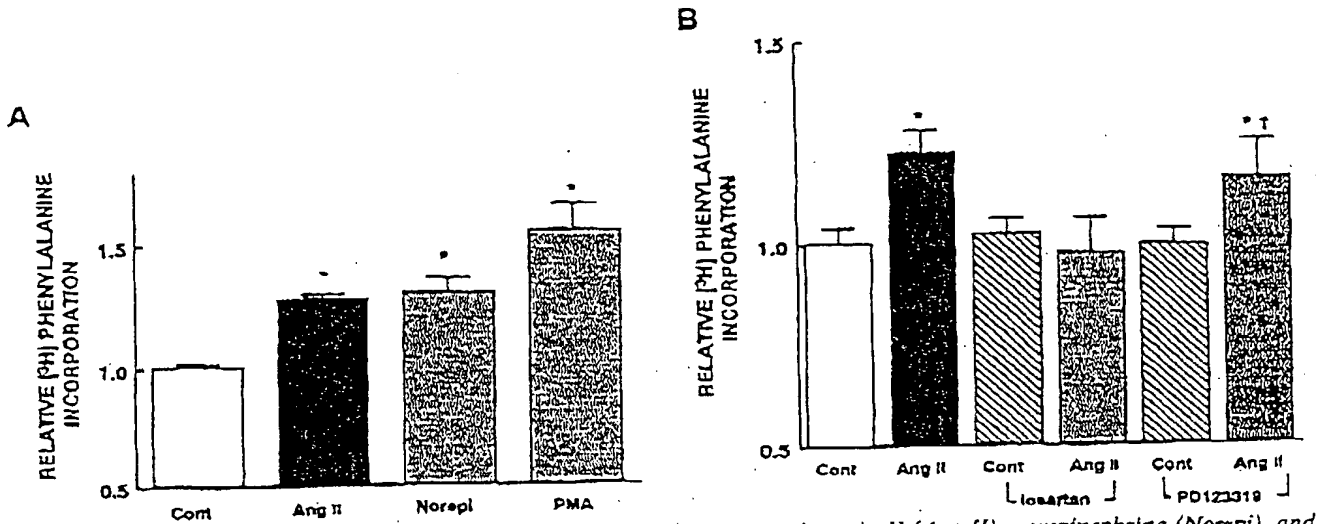

FIG 2. *A, Bar graph compares [³H]phenylalanine incorporation between angiotensin II (Ang II), norepinephrine (Norepi), and phorbol 12-myristate 13-acetate (PMA) in cardiac myocytes. Myocytes were stimulated with Ang II (10 nM), Norepi (20 μM), or PMA (1 μM) over 48 hours. Ang II and Norepi were applied every 12 hours to compensate for a decrease in agonist concentrations in the media due to degradation. Data are normalized to the mean counts per minute of control culture (Cont), which was set as 1. Data are mean+SEM obtained from four samples. *P<.01 vs control. B, Bar graph compares the effect of nonpeptide Ang II receptor antagonists on Ang II–induced [³H]phenylalanine incorporation in cardiac myocytes. For each antagonist, cells were pretreated with the antagonist for 30 minutes, and then Ang II (10 nM) was added in the presence of the antagonist. Ang II was added every 12 hours for 48 hours. Parallel control cultures received the antagonist without Ang II (hatched bars). Data are normalized to the mean of control culture without antagonist (Cont, white bar), which was set as 1. Data are mean+SEM obtained from four to five samples in each group. The concentrations of the antagonists used are as follows: losartan, 1 μM; and PD123319, 1 μM. *P<.05 vs Cont without drugs. †P<.05 vs Cont with PD123319.*

tein (Biomedical Technologies Inc, Stoughton, Mass) revealed that less than 2% of the cells took up acetylated low density lipoprotein (a marker for endothelial cells).[22] These preliminary characterizations of nonmyocyte culture suggest that the majority of the cells in nonmyocyte culture are likely to be fibroblasts, as defined by the lack of the markers for cardiac myocytes, smooth muscle, or endothelial cells.

Isolation and Northern Blot Analysis of RNA

Isolation of total cellular RNA and Northern blot analysis were performed as described previously.[20] The probes c-*fos*, c-*jun*, *Egr-1* (*Zif/268*), c-*myc*, skeletal α-actin, atrial natriuretic factor (ANF), and glyceraldehyde-3-phosphate dehydrogenase were used as described previously.[20] The following probes were also used: (1) *jun B*, a 1.8-kb *Eco*RI fragment of the mouse *jun B* cDNA,[23] (2) angiotensinogen, an *Eco*RI fragment of the rat angiotensinogen cDNA clone pGEM3,[24] and (3) transforming growth factor-β_1 (TGF-β_1), a *Sac I/Pvu* II fragment of the porcine TGF-β_1 clone pTGFβ33.[25] The relative amounts of a specific mRNA were quantified by laser densitometry of the corresponding autoradiograms in the linear response range of the x-ray films. The hybridization signals of specific mRNAs were normalized to those of glyceraldehyde-3-phosphate dehydrogenase mRNA to correct for differences in loading and/or transfer. The levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were not affected by Ang II (see "Results").

Incorporation of [³H]Phenylalanine

As an index of protein synthesis, [³H]phenylalanine incorporation was measured as described previously.[20] After incubation in serum-free medium for 24 hours, the cells were stimulated with Ang II (10 nM) for 48 hours in the presence of [³H]phenylalanine (10 μCi/mL) and unlabeled phenylalanine (0.36 mM) in the medium. The cells were washed with phosphate-buffered saline (PBS), and 10% trichloroacetic acid (TCA) was added at 4°C for 60 minutes to precipitate protein. For the control condition, parallel cultured cells were harvested at the same time course without Ang II stimulation. The precipitate was washed three times with 95% ethanol and then resuspended in 0.15N NaOH. Aliquots were counted in a scintillation counter. The results were expressed as counts per minute per dish.

Incorporation of [³H]Thymidine and Cell Counts

[³H]Thymidine uptake measurement and cell counts were performed as described.[20] For this experiment, BrdU was omitted from the culture medium. Cells were grown in a serum-free medium for 24 hours and then stimulated with 10 nM Ang II. After 18 hours, [³H]thymidine (5 μCi/mL) was added for 6 hours. Cells were then washed with PBS and harvested with 10% TCA. TCA-precipitable counts were measured as above.

BrdU Incorporation

A mixed culture of cardiac myocytes and nonmyocytes was prepared by omitting the preplating procedure. Cells were kept in a serum-free medium for 48 hours and then stimulated with Ang II (100 nM) for 24 hours. Control cultures were prepared without stimulation with Ang II. In both preparations, BrdU (10 μM) was added for the last 5 hours. Cells were fixed in methanol for 10 minutes at −20°C, rehydrated in PBS,

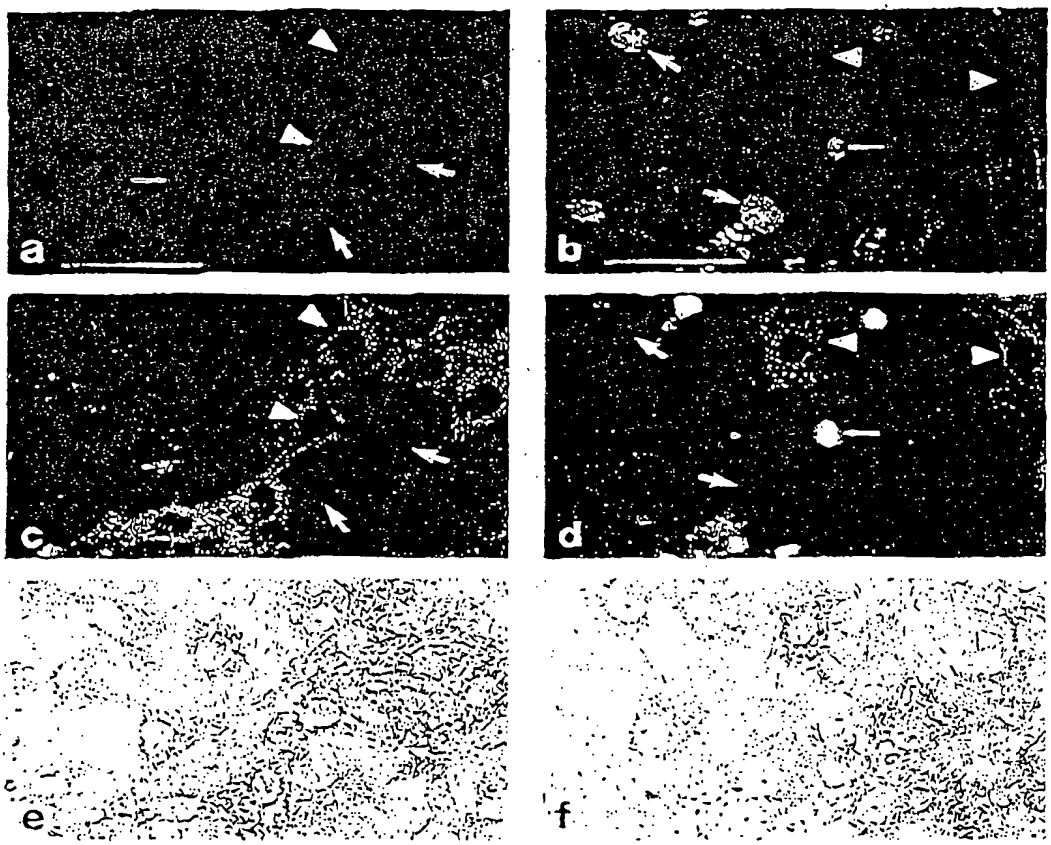

FIG 3. *Immunofluorescent staining of mixed culture of cardiac myocytes and nonmyocytes with anti-bromodeoxyuridine (anti-BrdU) and anti-myosin antibodies. Cells were kept in a serum-free medium for 48 hours and then stimulated with angiotensin II (Ang II, 10 nM) for 24 hours. Control culture was prepared without stimulation with Ang II. In both preparations, BrdU (10 μM) was added for the last 5 hours. a shows staining of control cells with anti-BrdU antibody conjugated with fluorescein isothiocyanate; c, staining of the same fields with anti-myosin antibody using a Texas red–conjugated secondary antibody; e, phase-contrast image of panels a and c (note that there is no nuclear BrdU staining either in myosin-positive cells [arrowheads] or myosin-negative cells [thick arrows]); b, staining of Ang II–stimulated cells with anti-BrdU antibody; d, staining of the same field with anti-myosin antibody; and f, phase-contrast image of panels b and d. Note that granular nuclear staining of BrdU was observed in a subset of myosin-negative cells (thick arrows). Note also that there is no BrdU staining in myosin-positive cells (arrowheads). To show BrdU-positive cells, a field containing more myosin-negative cells was shown. The bright spots (thin arrows) are artifacts corresponding to noncellular materials in the phase-contrast microscopic images. Bars=20 μm.*

and incubated in 2N HCl for 1 hour at 37°C. After neutralization in 0.1 M borate buffer (pH 8.5), cells were washed in PBS and processed for the immunofluorescent staining. Fluorescein isothiocyanate (FITC)–conjugated mouse monoclonal antibody against BrdU (Bu 5.1 FITC, IBL Research Products) and MF20 were used.[21] For the detection of MF20, Texas red–coupled goat antibodies to mouse immunoglobulins (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) were used. For double-label experiments, FITC-conjugated anti-BrdU antibody was applied after completion of the staining with MF20.

Immunohistochemistry

Immunofluorescence cell staining was performed as described previously.[20] For primary antibodies, rabbit serum 456 against c-*fos* (Medac, Hamburg, Germany) and MF20 were used. Secondary antibodies were FITC-conjugated or Texas red–coupled goat antibodies to immunoglobulins of rabbit or mouse (Jackson ImmunoResearch Laboratories). For double-label experiments, both primary and secondary antibodies were applied simultaneously.

Statistics

Data are given as mean±SEM. Statistical analysis was performed using analysis of variance and unpaired Student's *t* test as appropriate. Significance was accepted at $P<.05$.

Results

Ang II Causes Hypertrophy of Cardiac Myocytes

We examined the effects of Ang II on protein synthesis and the rate of DNA synthesis in the myocyte and nonmyocyte cell fractions (see "Materials and Methods") of primary cultured neonatal rat heart cells. In myocytes, Ang II (10 nM) caused a significant increase in protein synthesis as measured by [^{3}H]phenylalanine incorporation over 48 hours (Fig 1, A). The magnitude of increase in [^{3}H]phenylalanine incorporation induced

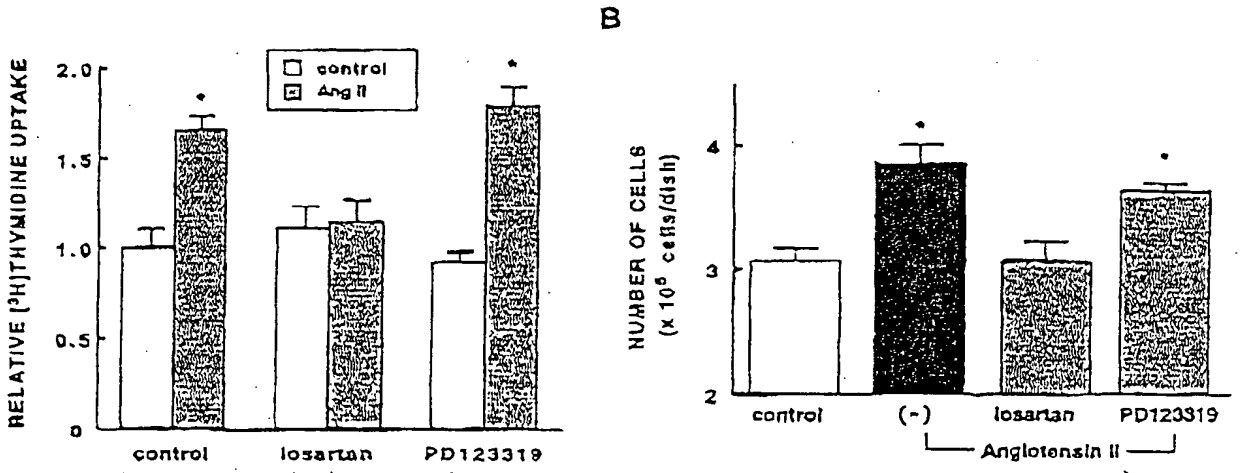

FIG 4. *Bar graphs showing the effect of nonpeptide angiotensin II (Ang II) receptor antagonists on Ang II–induced [3H]thymidine incorporation (A) and cell counts (B) in nonmyocytes. Cells were pretreated with each antagonist for 30 minutes and then stimulated with Ang II (10 nM) for 24 hours in the presence of the antagonist. Ang II (10 nM) was supplemented at 12 hours to compensate for decrease due to degradation by an endogenous angiotensinase. The concentrations of the antagonists used are as follows: losartan, 1 μM; and PD123319, 1 μM. A, [3H]Thymidine (5 μCi/mL) was added from 18 to 24 hours after addition of Ang II. Data are normalized to the mean counts per minute of the control value without drugs, which was set as 1. Data are mean+SEM obtained from five samples in each group. *P<.01 vs control without antagonists. B, Cell count was performed at 24 hours. Data are expressed as number of cells per dish and are mean+SEM obtained from five samples in each group. *P<.01 vs control without antagonist. Minus sign indicates Ang II alone.*

by Ang II (10 nM) was comparable to that induced by norepinephrine (20 μM) and was smaller than that induced by phorbol 12-myristate 13-acetate (1 μM) (Fig 2, A), two well-characterized hypertrophic stimuli for neonatal cardiac myocytes.[26-28] In contrast, Ang II (10 nM) did not increase DNA synthesis as measured by [3H]thymidine uptake over 24 hours (Fig 1, B). Lack of DNA synthesis in response to Ang II in myocytes was also confirmed by double immunostaining with an anti-myosin antibody and an anti-BrdU antibody (Fig 3, see below). These results suggest that Ang II has a hypertrophic effect (increase in protein synthesis without DNA synthesis) on neonatal rat cardiac myocytes.

AT_1 Receptor Mediates Ang II–Induced Hypertrophy of Myocytes

Recently, the presence of two Ang II receptor subtypes (AT_1 and AT_2) has been reported on the basis of binding site analyses using nonpeptide Ang II receptor antagonists. The prototypical antagonist of the AT_1 receptor is losartan (DuP 753) and that of the AT_2 receptor is PD123319.[29] We examined which Ang II receptor subtype was linked to protein synthesis in the neonatal rat cardiac myocyte. Neither losartan (1 μM) nor PD123319 (1 μM) significantly affected the basal level of protein synthesis in nonstimulated myocytes (Fig 2, B). Losartan completely suppressed the Ang II–induced increase in protein synthesis, whereas PD123319 did not suppress it significantly. The results suggest that the Ang II–induced increase in protein synthesis in cardiac myocytes is mediated by the AT_1 receptor.

Ang II Causes Hyperplasia of Nonmyocytes via AT_1 Receptors

In cardiac nonmyocyte culture (mostly fibroblasts, see "Materials and Methods"), Ang II (10 nM) treatment also caused a significant increase in [3H]phenylalanine incorporation over 48 hours (Fig 1, A). Interestingly, Ang II caused a significant increase in [3H]thymidine uptake over 24 hours in these cells (Fig 1, B), although the magnitude of increase caused by Ang II was 8% to 10% of that caused by 20% fetal calf serum (data not shown).

To confirm Ang II–induced DNA synthesis in nonmyocytes, the cells were labeled with the thymidine analogue BrdU, and a double immunofluorescent analysis was performed using anti-BrdU antibody and anti-sarcomeric myosin antibody (Fig 3). We deliberately used a mixed culture of myocytes and nonmyocytes for this analysis to examine both cell types in the same microscopic field. In control cells cultured in the serum-free medium for 48 hours, no staining was observed by anti-BrdU antibody in any of the myosin-positive cells (Fig 3, a and c; arrowheads). In myosin-negative cells (thick arrows), 6.5% of the cells were BrdU-positive (26 of 400 cells counted). When the cells were treated with Ang II for 24 hours, a clear nuclear staining pattern by BrdU antibody was observed in 32% of the myosin-negative cells (Fig 3, b and d; thick arrows and 128 of 400 cells counted) but never in myosin-positive cells (Fig 3, b and d; arrowheads) in the multiple fields examined. The mitogenic effect of Ang II on nonmyocytes was also confirmed by counting the number of cells (see below). These results suggest that Ang II has a mitogenic effect on nonmyocytes but not on myocytes.

We next examined which receptor subtype mediates the Ang II–induced mitogenic effect on nonmyocytes. As shown in Fig 4, A, losartan prevented Ang II–induced increase in [3H]thymidine uptake, whereas PD123319 did not. Similar results were observed when cell numbers were counted (Fig 4, B). Ang II caused a 30% increase in cell number over 24 hours, and this increase was com-

pletely prevented by losartan but not by PD123319 (Fig 4, B). Thus, the AT_1 receptor mediates Ang II–induced mitogenesis of cardiac nonmyocytes.

Induction of Immediate-Early Genes

A variety of stimuli that induce cardiac hypertrophic response, such as mechanical load, α_1- and β-adrenergic receptor agonists, endothelin-1, fibroblast growth factors, and TGF-β_1, have been shown to induce immediate-early (IE) genes as one of the earliest nuclear events (see References 20, 27, and 30-33; reviewed in Reference 34). It has been shown that Ang II causes the induction of IE genes such as c-*fos* and c-*myc* in vascular smooth muscle cells.[35,36] Therefore, we examined the effect of Ang II on the IE gene expression in myocytes and nonmyocytes. Since the expression pattern of the IE genes has been reported to be stimulus specific,[27] we examined the expression of three different classes of transcription factors: (1) c-*fos*, c-*jun*, and *jun B* (members of "leucine zipper" class genes),[37] (2) *Egr-1* (a "zinc finger" class gene),[38] and (3) c-*myc* (a "helix-loop-helix"–containing gene).[39] Representative Northern blots are shown in Fig 5. In both myocytes and nonmyocytes, Ang II induced c-*fos*, c-*jun*, *jun B*, *Egr-1*, and c-*myc*. IE genes c-*fos*, *jun B*, and *Egr-1* showed a peak induction at approximately 30 minutes, whereas c-*jun* and c-*myc* showed a later peak at approximately 30 minutes to 1 hour. The duration of augmented expression of c-*fos* was shorter than that of the others, reverting to the control level at 2 hours, whereas that of the others showed moderately elevated expression even at 2 hours.

Recently Roux et al[40] reported that, in the absence of serum, the Fos protein could not be translocated into nucleus and stayed in cytoplasm in rat embryonic fibroblasts and mouse fibroblast cell lines. Therefore, we examined whether growing the cardiac myocytes and nonmyocytes in serum-free conditions actually leads to translocation of Fos protein to the nucleus after its synthesis in the cytoplasm in response to Ang II stimulation. Double immunofluorescence staining was performed on a mixed cell culture using anti-Fos and anti–sarcomeric myosin antibodies. Both myosin-positive cells (Fig 6, a through c; arrowheads) and myosin-negative cells (arrows) expressed Fos protein 1 hour after treatment with Ang II (Fig 6, a). No specific Fos signals were observed in nontreated control culture (Fig 6, d). Thus, in cardiac myocytes and nonmyocytes, Ang II induces translocation of Fos to the nucleus in the absence of serum. Induction of Fos protein was a transient response because little Fos signal was detectable 3 hours after Ang II treatment (data not shown).

Induction of c-fos by Ang II Is Mediated Primarily by AT_1 Receptors

Ang II induced c-*fos* expression in a dose-dependent manner in cardiac myocytes (Fig 7, A). The induction of c-*fos* was detected at 10 pM, and maximum induction was observed at approximately 100 nM. The dose-response relation of the c-*fos* induction as quantitated

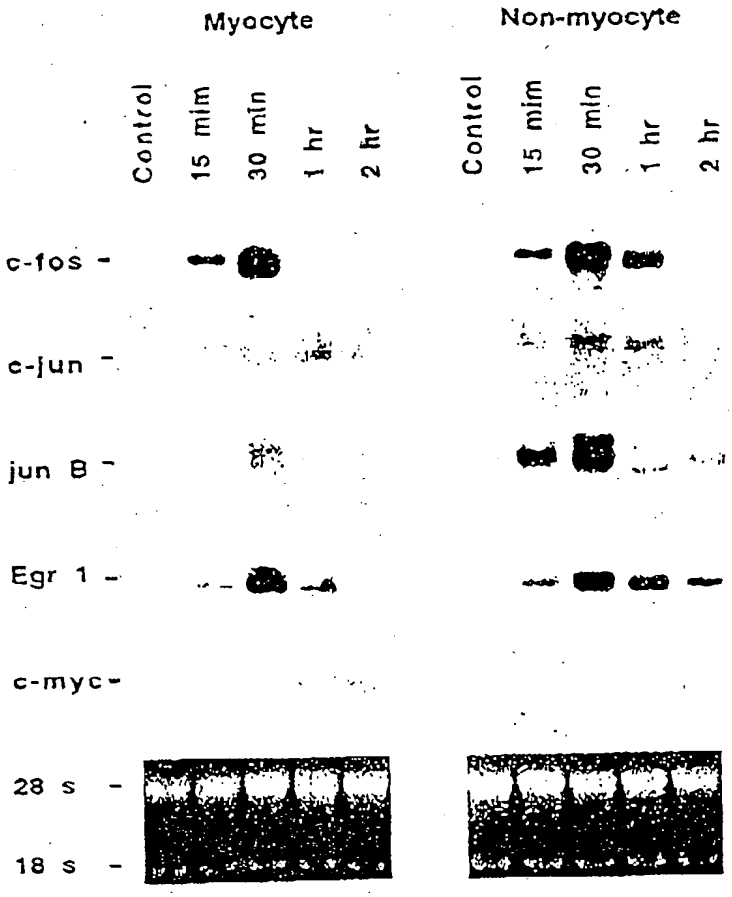

FIG 5. *Angiotensin II (Ang II)–induced expression of immediate-early genes in myocytes and nonmyocytes in the neonatal rat heart. Representative Northern blots of myocytes (left) and nonmyocytes (right) are shown. The same blots were hybridized serially by different probes to demonstrate the different kinetics of each immediate-early gene. Myocyte and nonmyocyte fractions were prepared as described in the text. Cells were stimulated with Ang II (100 nM) for the times indicated on the top. Ethidium bromide staining of 18S and 28S RNA shown below showed that an equal amount of RNA was loaded in each lane. The serial hybridization resulted in higher background and lower signal intensity as seen in c-jun and c-myc probes that were hybridized after c-fos and EGR-1 probes. However, in other blots, significant induction of c-jun and c-myc by Ang II was observed. Similar results were obtained from two additional experiments.*

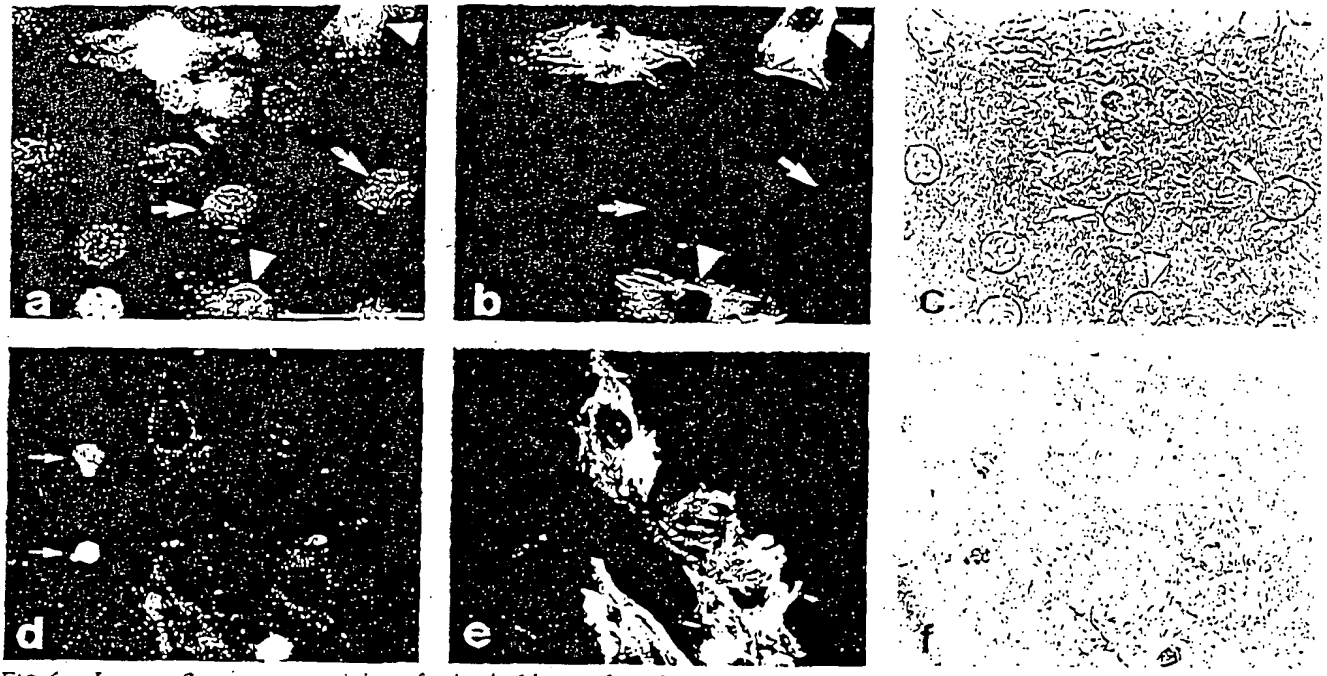

FIG 6. *Immunofluorescence staining of mixed culture of cardiac myocytes and nonmyocytes with anti-Fos and anti-myosin antibodies. Cells were plated on glass coverslips in serum-free conditions for 48 hours. After a 60-minute treatment with angiotensin II (100 nM), the cells were fixed, and a double staining was performed. a shows staining by the anti-Fos antibody using a fluorescein isothiocyanate–conjugated secondary antibody. Granular nuclear staining was observed in most of the cells in the field. b shows staining of the same field by the anti-sarcomeric myosin antibody using a Texas red–conjugated secondary antibody. c is a phase-contrast image of panels a and b. Note that both myosin-positive cells (arrowheads) and myosin-negative cells (arrows) expressed Fos protein in the nucleus in response to angiotensin II. d shows staining of control cells with anti-Fos antibody. e shows staining of the same field as in panel d by the anti-sarcomeric myosin antibody. f is a phase-contrast image of panels d and e. The bright spots (thin arrows) are artifacts. Bar=20 μm.*

by laser densitometry showed a half-maximum concentration (EC_{50}) of 1 to 2 nM. This is consistent with a known K_d of Ang II to its receptor in cardiac myocytes.[41]

Figure 7, B, is a representative Northern blot showing the effects of nonpeptide Ang II receptor antagonists on Ang II (1 nM)–induced c-*fos* expression. The AT_1 receptor antagonist losartan (100 nM) almost completely suppressed c-*fos* expression, but the same concentration (100 nM) of the AT_2 antagonist PD123319 did not show significant inhibition. At 100-fold higher concentration (10 μM), PD123319 partially suppressed Ang II–induced c-*fos* expression. This effect may be due to a partial block of the AT_1 receptor at this concentration of PD123319, although we cannot formally rule out the possibility that a minor component of Ang II–induced c-*fos* expression may be mediated by the AT_2 receptor.

Effects of Angiotensin Metabolites on c-fos Induction

Recently, the existence of the local renin-angiotensin system has been reported in various tissues, including the rat heart.[3-6] To identify the biological activity of several metabolites of the renin-angiotensin system, we examined c-*fos* inducibility by exogenously applying angiotensin-related peptides to the cardiac myocyte. Fig 8 shows a representative Northern blot. Ang I (100 nM) and its aminopeptidase-cleavage product [des-Asp^1]Ang I (100 nM) induced c-*fos* expression (lanes 8 and 10). However, when Ang I and [des-Asp^1]Ang I were applied with the ACE inhibitor captopril (10 μM) to prevent their conversion into Ang II and angiotensin III (Ang III), respectively, they did not induce c-*fos* (lane 9 and data not shown). This suggests that not only is there an endogenous ACE-like activity in cultured cardiac cells but that Ang I and [des-Asp^1]Ang I require this ACE-like activity to induce c-*fos* in cardiac myocytes.

Among the Ang II degradation products, Ang III (10 nM) induced c-*fos* expression almost as potently as did Ang II (Fig 8, lane 4). However, the aminopeptidase cleavage products Ang II-(3-8) (100 nM) and Ang II-(4-8) (100 nM) did not induce c-*fos* (lanes 5 and 6). [Sar^1,Ile^8]Ang II (100 nM), a nonselective competitive inhibitor of Ang II, did not induce c-*fos* by itself (lane 3) and completely prevented the c-*fos* induction by Ang II (10 nM) (lanes 1 and 2). As expected, captopril did not prevent c-*fos* induction by Ang II or Ang III (data not shown).

Ang II Causes Induction of Fetal Genes, Angiotensinogen Gene, and TGF-β1 Gene

It is known that cardiac hypertrophy in vivo and in vitro is accompanied by changes in the muscle phenotype characterized by the expression of "fetal"-type genes, such as skeletal α-actin and the ventricular expression of ANF.[20,26,31,32,34] Therefore, we examined the expression of these fetal genes in response to Ang II (Fig 9, A). Although ANF and skeletal α-actin mRNAs were detectable under control conditions in these neo-

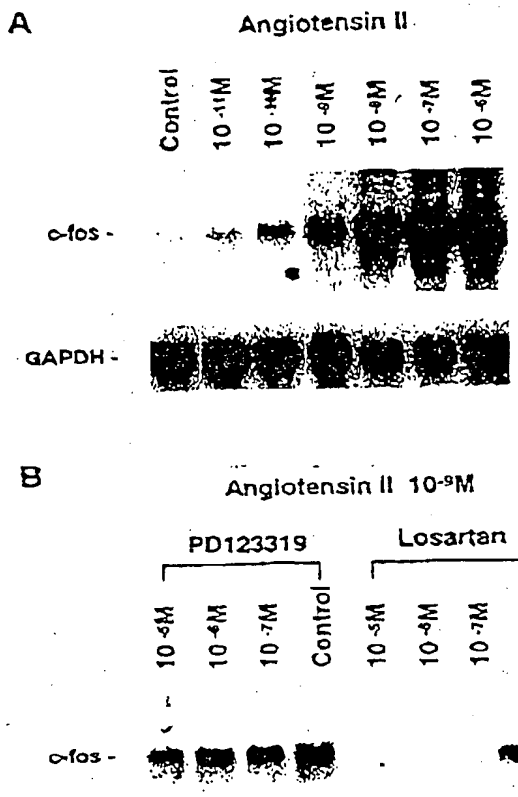

FIG 7. *A, Representative Northern blot shows dose-response relation of angiotensin II–induced c-fos expression. Cardiac myocytes were treated with angiotensin II for 30 minutes with the concentrations indicated on the top. Hybridization of the same blot with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe indicated that equal amounts of RNA were loaded in each lane (bottom). Similar results were obtained from two additional experiments. B, Representative Northern blot shows effect of nonpeptide angiotensin II receptor antagonists on angiotensin II–induced c-fos expression. Cardiac myocytes were pretreated with each antagonist for 30 minutes and then stimulated with angiotensin II (1 nM) for 30 minutes in the presence of the antagonist with the concentrations indicated. Hybridization with GAPDH probe showed equal signals in each lane (not shown). Angiotensin II–induced c-fos expression was fully inhibited by 10^{-7} M of losartan, an AT_1 receptor antagonist, but not by 10^{-7} M of PD123319, an AT_2 antagonist. Similar results were obtained from two additional experiments.*

natal ventricular myocytes, 6 to 24 hours of treatment with Ang II significantly increased the expression of ANF and skeletal α-actin genes in a time-dependent fashion.

Some growth factors stimulate or repress transcription of their own gene and of other growth factor genes, which provides a positive- or negative-feedback regulation of cell growth.[25] It has been shown that Ang II increases the amount of angiotensinogen mRNA in the rat liver.[42] Therefore, we examined whether Ang II affects the accumulation of angiotensinogen mRNA in cardiac myocytes. We also examined levels of TGF-β_1

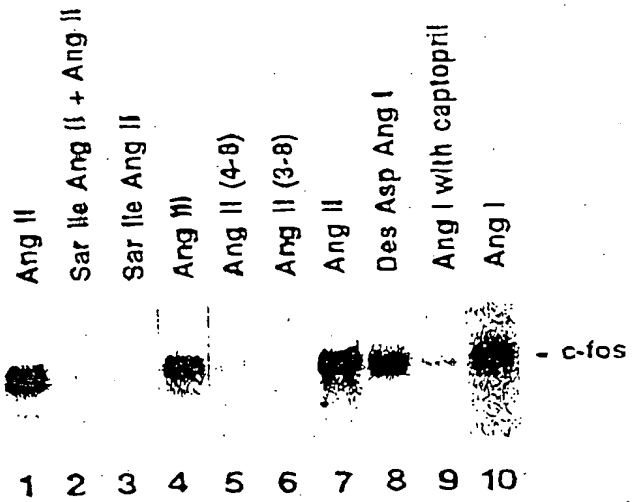

FIG 8. *Induction of c-fos expression by angiotensin-related peptides. Ang I indicates angiotensin I (100 nM); Des Asp Ang I, [des-Asp¹]angiotensin I (100 nM); Ang II, angiotensin II (10 nM); Sar Ile Ang II, [Sar¹,Ile⁸]angiotensin II (100 nM); Ang III, angiotensin III (10 nM); Ang II (3-8), angiotensin II-(3-8) (100 nM); and Ang II (4-8), angiotensin II-(4-8) (100 nM). The concentration of captopril was 10 μM. Cardiac myocytes were treated with exogenously applied angiotensin-related peptides for 30 minutes. For lane 2 (Sar Ile Ang II + Ang II) and lane 9 (Ang I with captopril), cells were pretreated with [Sar¹,Ile⁸]Ang II or captopril for 30 minutes, and Ang II and Ang I were applied in the presence of [Sar¹,Ile⁸]Ang II and captopril, respectively. Hybridization with the glyceraldehyde-3-phosphate dehydrogenase probe showed equal amounts of RNA loaded in each lane (not shown). Similar results were obtained from two to four additional experiments.*

mRNA in response to Ang II stimulation because TGF-β_1 is known to be a potent inducer of the fetal genes in neonatal rat cardiac myocytes.[33] As shown in Fig 9, B, a significant induction of angiotensinogen mRNA and TGF-β_1 mRNA was observed 6 hours after Ang II treatment, and this effect appeared more pronounced at 24 hours.

Increased Expression of "Late" Genes Is Mediated by AT_1 Receptors

We next examined whether increase in "late" genes by Ang II stimulation was mediated by AT_1 or AT_2 receptors. Ang II–induced increases in mRNAs encoding skeletal α-actin, ANF, TGF-β_1, and angiotensinogen were significantly suppressed by losartan but not by PD123319 (Fig 10 and data not shown). These results suggest that induction of late genes is primarily mediated by the AT_1 receptor subtype.

Discussion

We have analyzed the effects of Ang II in primary cultured cardiac myocytes and nonmyocytes (mostly fibroblasts). The major findings are as follows: First, Ang II causes hypertrophy of cardiac myocytes and hyperplasia of cardiac nonmyocytes. Second, both hypertrophy of myocytes and hyperplasia of nonmyocytes are mediated by the AT_1 receptor. Third, Ang II induces

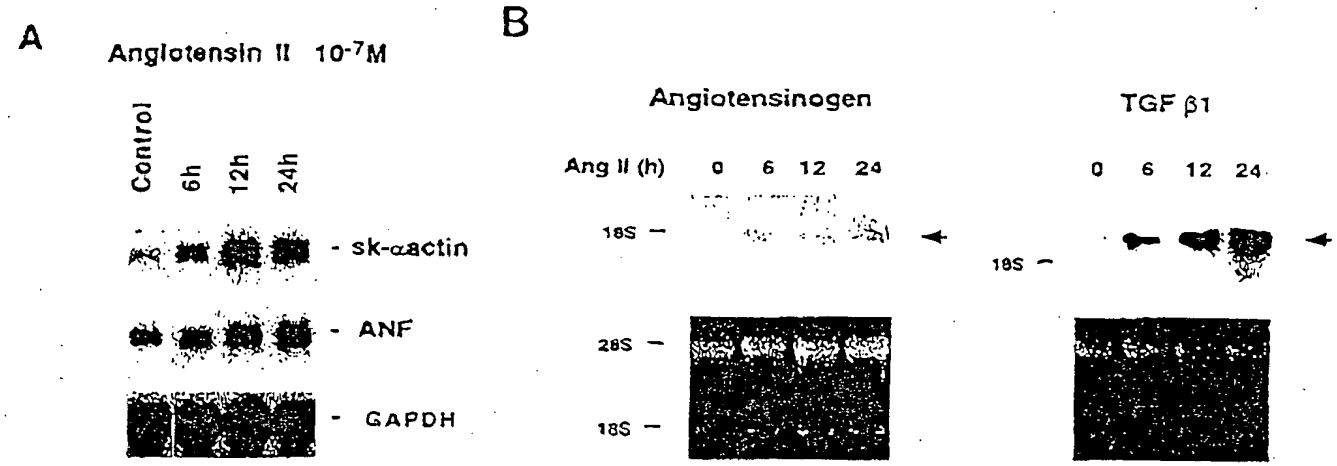

FIG 9. *A, Representative Northern blot shows induction of atrial natriuretic factor (ANF) and skeletal α-actin (sk-αactin) gene by angiotensin II (Ang II). Ang II (100 nM) was added to the cardiac myocytes every 6 hours to compensate for degradation by an endogenous angiotensinase. Hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe showed equal amounts of RNA in each lane. Similar results were obtained from four additional experiments. B, Representative Northern blots show induction of angiotensinogen and transforming growth factor-β1 (TGF β1) gene by Ang II. Ang II (100 nM) was added to the cardiac myocytes every 6 hours for the times indicated above. Hybridization with GAPDH probe (not shown) and ethidium bromide staining of 18S and 28S RNA (bottom) showed equal amounts of RNA in each lane. Similar results were obtained from three additional experiments.*

the expression of a number of IE genes, such as *c-fos, c-jun, jun B, Egr-1,* and *c-myc,* in both myocytes and nonmyocytes. Fourth, Ang II induces the "fetal program" (induction of skeletal α-actin and ANF) and induces expression of the angiotensinogen gene and TGF-β1 gene. Fifth, Ang II–induced changes in IE genes and late genes are primarily mediated by the AT_1 receptor.

The increase in the rate of protein synthesis by Ang II in the neonatal rat cardiac myocytes seems to be compatible with that in chick heart cells reported by Baker and Aceto[15] (40% above the control value over 120 hours). However, the Ang II–induced increase in the protein synthesis in cardiac myocytes was smaller than that in the vascular smooth muscle cells reported by Berk et al.[9] It is possible that Ang II has a greater growth effect on the smooth muscle cells than on cardiac myocytes. However, the experiment of Berk et al was carried out in the presence of 0.4% calf serum; thus, a potential synergistic effect with residual serum-derived growth factors cannot be excluded. Moreover, Geisterfer et al[8] have reported that in rat aortic smooth muscle cells Ang II (1 μM) increases the rate of protein synthesis by approximately 25% over 48 hours, which is compatible with our results.

It is of interest that Ang II was mitogenic to cardiac nonmyocytes, whereas it was strictly hypertrophic to cardiac myocytes. The latter fact may not be solely due to the terminally differentiated state of neonatal cardiac myocytes, because at this developmental stage some myocytes are reported to still retain their ability to synthesize DNA in response to serum stimulation.[43] It would also be interesting to determine whether the cell cycle regulatory genes, such as cyclins and cyclin-dependent kinases, are regulated differently in response to Ang II in myocytes and nonmyocytes. However, it should be emphasized that Ang II is not as strong a mitogen as fetal calf serum, because the Ang II–induced increase in thymidine incorporation is only 8% to 10% of that seen with fetal calf serum. Ang II has been shown to be mitogenic to Swiss 3T3 cells[44] and some types of vascular smooth muscle cells.[11,12]

van Krimpen et al[45] reported that increased DNA synthesis in interstitial cells after myocardial infarction was inhibited by an ACE inhibitor independent of its effect on afterload changes. Weber and Brilla[46] found that Ang II causes fibrosis and increased collagen deposition in the cardiac interstitium. This effect was prevented by an ACE inhibitor and by an aldosterone inhibitor. In our in vitro system, the mitogenic effect of Ang II on nonmyocytes is most likely a direct effect of Ang II, rather than being mediated by aldosterone, because the latter hormone is not known to be produced in cardiac cells. Therefore, the beneficial effects of ACE inhibitors in myocardial remodeling after myocardial infarction may be due to a decreased production of Ang II, although it is not possible to rule out other nonspecific effects of ACE inhibitors in vivo.

It has been shown that both AT_1 and AT_2 receptors are expressed in rat neonatal cardiac myocytes and possibly in nonmyocytes.[47] Our pharmacological studies indicate that all aspects of Ang II–induced myocyte hypertrophy examined (increase in protein synthesis and induction of IE genes and late genes) are primarily mediated by the AT_1 receptor subtype. Our results also demonstrate that the mitogenic response of nonmyocytes is mediated by the AT_1 receptor. The importance of the AT_1 receptor in normal growth of newborn pig hearts in vivo has recently been demonstrated.[16] At present, physiological roles of the AT_2 receptor are not known in cardiac cells.

Recently, cDNAs encoding the AT_1 receptors have been cloned.[48,49] The deduced amino acid sequence predicts seven membrane spanning regions, typical of G protein–coupled receptors. The receptors for other hypertrophic stimuli, endothelin-1 and α-adrenergic ag-

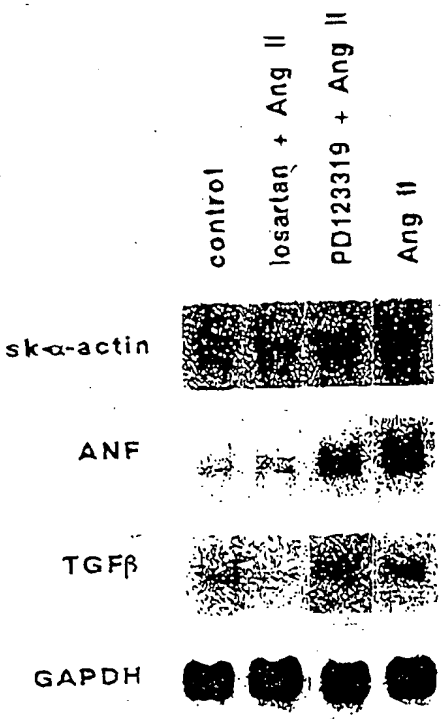

FIG 10. *Representative Northern blot shows effect of nonpeptide angiotensin II (Ang II) receptor antagonists on Ang II–induced expression of the fetal cardiac genes and growth factor genes. Cardiac myocytes were pretreated with each antagonist for 30 minutes and then stimulated with Ang II (100 nM) for 24 hours in the presence of the antagonist. Ang II was added every 6 hours. The concentrations of the antagonists used are as follows: losartan, 1 μM; and PD123319, 1 μM. Hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe indicated equal amounts of RNA in each lane. Note that the induction of skeletal α-actin (sk-α-actin), atrial natriuretic factor (ANF), and transforming growth factor-β_1 (TGFβ) by Ang II was inhibited by losartan but not significantly by PD123319. Similar results were obtained from two additional experiments.*

onists, also have similar structure. These receptors are believed to couple to G_q-type G proteins (reviewed in Reference 50). The phenotypic resemblance of Ang II–induced hypertrophy to that induced by endothelin-1 or α-adrenergic agonists may be due to the shared intracellular signaling in response to these agonists. On the other hand, TGF-β_1 and basic fibroblast growth factor also cause similar phenotypic changes in cardiac myocytes.[33] However, the initial signal transduction pathways by these agonists are different from those of G protein–coupled receptors.[25] It remains to be determined at what point the convergence of the signaling occurs in response to various hypertrophic stimuli.

In the present study, we have used neonatal cardiac myocytes and nonmyocytes. There remains a possibility that the response of neonatal cardiac myocytes and nonmyocytes may differ from that of adult cells. It has been reported that the responses to α-agonists are different between neonatal and adult myocytes.[51] Although several in vivo studies using ACE inhibitors suggest that Ang II may act as a growth factor for adult heart,[4-6] a direct proof for this hypothesis awaits a study using cultured adult myocytes. Interestingly, Moalic et al[52] have reported that infusion of Ang II did not induce c-*fos* or c-*myc* in an isolated perfused adult rat heart, presumably because of the absence of Ang II receptors in the adult rat heart. Very recently, however, Dostal and Baker[53] have reported that chronic infusion of Ang II causes cardiac hypertrophy and that this is prevented by losartan in adult rat hearts. It remains to be determined whether our in vitro results using neonatal cells may apply for the adult heart in vivo.

It is of interest that Ang II increases the expression of the angiotensinogen gene. This raises the possibility that Ang II may initiate a positive-feedback regulation of cardiac growth. Ang II also increases expression of the TGF-β_1 gene. TGF-β_1 is known to induce "fetal" genes in cardiac myocytes[33] and enhance the synthesis of the extracellular matrix by fibroblasts.[25,54] Ang II has been shown to induce platelet-derived growth factor[25] and TGF-β_1[55] in smooth muscle cells. It is likely that Ang II may induce other growth factor genes in cardiac myocytes as well. It is possible that the mitogenic effect of Ang II on nonmyocytes may be due to an enhanced autocrine/paracrine production of growth factors by Ang II stimulation.

In summary, we have demonstrated that Ang II causes hypertrophy of cardiac myocytes and mitogenesis of nonmyocytes (primarily fibroblasts) via AT_1 receptor stimulation. Elucidation of the mechanisms of Ang II–induced cardiac growth deserves further investigation because Ang II is clearly emerging as one of the most important mediators of cardiac hypertrophy in vivo.

Acknowledgments

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Angiotensin II formation from ACE and chymase in human and animal hearts: methods and species considerations

EDUARDO BALCELLS, QING C. MENG, WALTER H. JOHNSON, JR., SUZANNE OPARIL, AND LOUIS J. DELL'ITALIA

Birmingham Veteran Affairs Medical Center and Division of Cardiovascular Disease, Department of Medicine, Vascular Biology and Hypertension Program, and Division of Pediatric Cardiology, Department of Pediatrics, University of Alabama, Birmingham, Alabama 35294

Balcells, Eduardo, Qing C. Meng, Walter H. Johnson, Jr., Suzanne Oparil, and Louis J. Dell'Italia. Angiotensin II formation from ACE and chymase in human and animal hearts: methods and species considerations. *Am. J. Physiol.* 273 (*Heart Circ. Physiol.* 42): H1769–H1774, 1997.—The current study examined the contributions of angiotensin-converting enzyme (ACE) vs. chymase to angiotensin II (ANG II) generation in membrane preparations from left ventricles of humans, dogs, rabbits, and rats and from total heart of mice. ACE and chymase activity were measured in membrane preparations extracted with low or high detergent (LD and HD, respectively) concentrations. We hypothesized that ACE, which is membrane bound in vivo, would be preferentially localized to the HD preparation, whereas chymase, which is localized to the cytoplasm and cardiac interstitium, would be localized to the LD preparation. In human heart, ACE activity was 16-fold higher in the HD than in the LD preparation, whereas chymase activity was 15-fold higher in the LD than in the HD preparation. Total ANG II formation was greater in human heart [15.8 ± 3.4 (SE) μmol ANG II·g^{-1}·min^{-1}] than in dog, rat, rabbit, and mouse hearts (3.90 ± 0.36, 0.41 ± 0.02, 0.61 ± 0.07, and 1.16 ± 0.08 μmol ANG II·g^{-1}·min^{-1}, respectively, $P < 0.05$, by analysis of variance). ANG II formation from ACE was higher in mouse heart (1.09 ± 0.05 μmol ANG II·g^{-1}·min^{-1}, $P < 0.001$) than in rabbit, human, dog, and rat hearts (0.55 ± 0.06, 0.34 ± 0.01, 0.32 ± 0.06, and 0.31 ± 0.02 μmol ANG II·g^{-1}·min^{-1}, respectively). In contrast, chymase activity was higher in human heart (15.3 ± 3.4 μmol ANG II·g^{-1}·min^{-1}) than in dog, rat, rabbit, and mouse hearts (3.59 ± 0.29, 0.10 ± 0.01, 0.06 ± 0.01, and 0.07 ± 0.01 μmol ANG II·g^{-1}·min^{-1}, respectively). Our results demonstrate important species differences in the pathways of intracardiac ANG II generation. Chymase predominated over ACE activity in human heart, accounting for extremely high total ANG II formation in human heart compared with dog, rat, rabbit, and mouse hearts.

angiotensin-converting enzyme; detergent renin-angiotensin system

COMPONENTS OF THE renin-angiotensin system (RAS) have been demonstrated in the heart by biochemical, immunohistochemical, and molecular biologic techniques (12) and have been shown to be upregulated in hearts that have developed pressure and volume overload-induced hypertrophy and failure (5, 9, 17, 22). Thus there is increasing evidence that angiotensin II (ANG II) formation in the heart is mediated by a local RAS, acts independently of the circulating RAS, and is upregulated by hemodynamic stress. However, most research on the intracardiac RAS in the basal state and under conditions of stress has been performed in rodent models, and the relevance to humans has been questioned, especially since there are multiple ANG II-forming pathways in cardiac tissue that vary among species (4, 16). In particular, a serine protease with extremely high affinity for ANG I, "chymostatin-sensitive angiotensin-generating enzyme," has recently been identified in the human (25), dog (1, 3), and baboon (6) heart, but not in the rodent heart (7).

Human "heart chymase" has been purified, cloned, and sequenced from the human heart (26). It is insensitive to angiotensin-converting enzyme (ACE) inhibition, and the catalytic activity for conversion of ANG I to ANG II is 20-fold higher for chymase than for ACE (8). However, there has been controversy regarding the ANG II-forming capacity of chymase vs. ACE in human heart tissue extracts. Urata and co-workers (25) reported that this enzyme represents ~90% of the ANG II-forming capacity in human heart tissue extracts solubilized with low detergent concentration (0.01% Triton), suggesting that ACE is not the major ANG II-forming enzyme in the human left ventricle in vitro. In contrast, Zisman and co-workers (29) demonstrated >80% ANG II formation from ACE in human heart tissue extracts solubilized with high detergent concentration (0.6% Triton) and dialysis. However, Wolny and co-workers (27) recently showed that solubilization with high concentrations of detergent and dialysis result in a major loss of chymase activity during sample preparation, thus providing a possible explanation for underestimating chymase-mediated ANG II-forming activity in the study of Zisman and co-workers. Taken together, these studies demonstrate that ANG II-forming capacities of ACE and chymase in cardiac tissue in vitro differ according to the method used to process the tissue.

The controversy over the predominance of ACE- vs. chymase-induced ANG II formation in heart tissue extracts may be related to the localization of chymase and ACE in the heart. ACE is bound to cell membranes of endothelial cells (10), whereas chymase is stored in vesicles in the intracellular compartment of mast cells and other types of interstitial cells in the heart (24). The presence of chymase in the heart has raised important questions regarding the origin of ANG II and the mechanism of action of ACE inhibitors, not only in the human heart but also in animal models of hypertrophy and heart failure. ANG II formation from ACE and chymase has not been systematically compared in the human heart and in the hearts of various animal species. Thus the purpose of the current investigation was to quantitate and compare ANG II-forming capacity of ACE and chymase in heart tissue from human, dog, rat, mouse, and rabbit. We hypothesized that ACE,

which is membrane bound in vivo, would be preferentially localized to the preparation with the high detergent concentration, whereas chymase, which is localized to the cytoplasm and cardiac interstitium, would be localized to the preparation with the low detergent concentration.

METHODS

Tissue Procurement

Normal human donor hearts (n = 5) not suitable for transplantation were obtained at the time hearts were harvested for organ donation at the University of Alabama at Birmingham. All hearts were kept in cold cardioplegia solution from the time of removal, and tissue from the left ventricular free wall was frozen in liquid nitrogen within 5–10 min. Hearts from adult mongrel dogs (n = 5, 20–25 kg body wt) and rabbits (n = 3, 3–4 kg body wt; New Zealand White, Myrtles, Memphis, TN) were obtained after a deep surgical plane of anesthesia was induced with isoflurane inhalation anesthesia for the thoracotomy. Hearts were arrested with a lethal dose of KCl, removed from the chest, rapidly cooled in ice-cold phosphate buffer, and placed on a stainless steel tray on ice. All animal hearts were dissected free of major blood vessels, cardiac valves, atria, and right ventricle. Tissue from the left ventricular midwall was frozen in liquid nitrogen within 2–5 min and stored at −80°C.

Male Sprague-Dawley rats (n = 8, mean weight 300 g) and male CD-1 mice (n = 6, mean weight 25 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA) and decapitated before removal of the heart. Rat hearts were dissected free of cardiac valves, atria, and right ventricle. The left ventricular free wall was frozen in liquid nitrogen within 2–8 min and stored at −80°C. Mouse hearts were frozen in toto in liquid nitrogen within 2–8 min after decapitation and stored at −80°C.

The protocol was approved by the Institutional Review Board for Human Use of the University of Alabama at Birmingham and by the Animal Services Committees at the University of Alabama at Birmingham.

Cardiac Membrane Preparation

Low detergent concentration. Membranes were prepared at 4°C in a manner similar to that previously described in our laboratory (1, 3) and by Urata and co-workers (25). Frozen heart tissue was homogenized with a Polytron (Fisher Scientific, Pittsburgh, PA) for 60 s in 100 mM potassium phosphate buffer (PBS), pH 7.4 in a 10:1 volume ratio, then centrifuged for 30 min at 44,000 g at 4°C with 0.01% Triton X-100. This procedure was repeated three times, and the supernatant and pellet fractions were collected as fraction *supernatant 1* (*S1*) and *pellet 1* (*P1*), respectively (Fig. 1). ANG II-forming activity in *P1* was obtained from an aliquot of the resuspended tissue pellet in 100 mM PBS (pH 8.3).

High detergent concentration. The pellet fraction (*P1*), prepared as described above, was resuspended in 1 ml of PBS buffer (pH 8.3) with 0.6% Triton X-100, vortexed for 3 min, and mechanically agitated for 4 h at 4°C. The tissue suspension was then centrifuged at 40,000 g for 30 min, and the supernatant and pellet fractions were collected as fractions *S2* and *P2*, respectively. ANG II-forming activity in *P2* was obtained from an aliquot of the resuspended tissue pellet in 100 mM PBS (pH 8.3), whereas an aliquot of the *S2* fraction was assayed without further processing.

ANG II-Forming Activity From ACE and Heart Chymase

Aliquots (10 μl) were taken from each of the above fractions and preincubated for 30 min at room temperature with an enzyme inhibitor solution specific to chymase-like or ACE activity assays. For ANG II-forming chymase activity assays, the inhibitor solution contained 2 mM EDTA, 100 μM captopril (Sigma Chemical, St. Louis, MO), 1 mM *o*-phenanthroline, and 20 μM aprotinin with and without 100 μM chymostatin. For ANG II-forming ACE activity assays, the inhibitor solution contained 1 mM *o*-phenanthroline, 20 μM aprotinin, and 100 μM chymostatin with and without 100 μM captopril. Samples were then incubated for 60 min at 37°C with 600 μM ANG I (Sigma Chemical) in 100 mM phosphate buffer (pH 8.8) solution containing 300 mM NaCl and 10^{-4} M ZnCl (omitted from chymase activity assays) to a final total volume of the reaction assay of 250 μl. Reactions were terminated by addition of ice-cold ethanol in a 1:3 (vol/vol) sample-to-ethanol ratio.

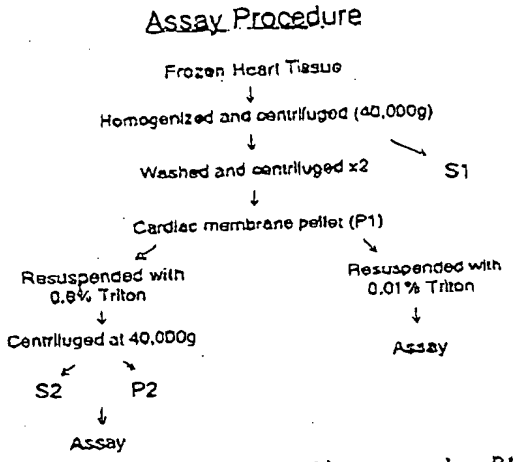

Fig. 1. Protocol for tissue extraction of heart samples. *P1* and *P2*, pellets; *S1* and *S2*, supernatants.

Generated ANG II was quantitated using a reverse-phase Alltima 5-μm phenyl-high-performance liquid chromatography column (Alltech Associates, Deerfield, IL), as previously performed in our laboratory (1, 3). The peak area corresponding to a synthetic ANG II standard was integrated to calculate absolute ANG II formation. ANG II-forming activity from ACE was defined as the captopril-inhibitable ANG II formed, whereas chymase activity was defined as the chymostatin-inhibitable ANG II formed. ACE and chymase-like activities are expressed as moles of ANG II formed per gram of protein per minute and protein content was determined by the method of Lowry and co-workers (14).

Statistics

Values are means ± SE. Analysis of variance with Newman-Keuls post hoc comparison was used to compare ANG II-forming capacity from ACE and chymase in *S1*, *S2*, and *P2* of HD and LD cardiac membrane preparations and total ANG II formation across species. $P < 0.05$ was required to reject the null hypothesis.

RESULTS

ANG II Formation From ACE and Chymase in the Human Heart

ANG II formation from chymase was higher in the low-detergent membrane fractions (*P1* and *P2*: 15.3 ± 3.4 and 13.3 ± 2.9 μmol·g protein^{-1}·min^{-1}) than in the high-detergent membrane fraction (*S2*: 0.96 ± 0.21 μmol·g protein^{-1}·min^{-1}, $P < 0.001$; Fig. 2*A*). In con-

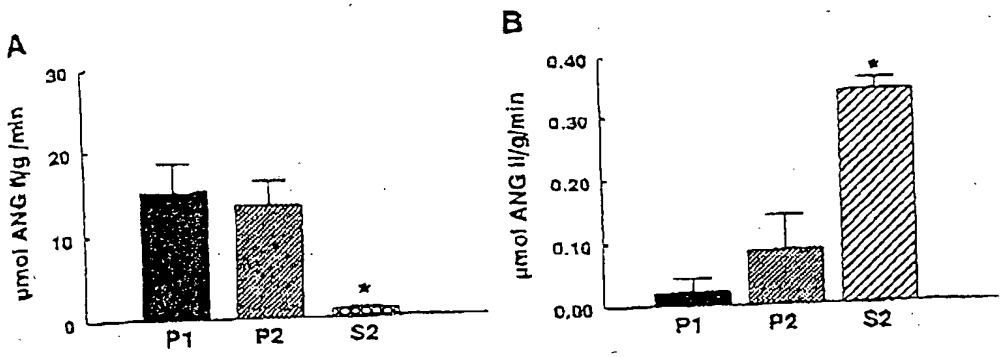

Fig. 2. ANG II formation from chymase (*A*) and angiotensin-converting enzyme (*B*) in low- (0.01% Triton) and high-detergent extraction (0.6% Triton) cardiac membrane preparations of human heart tissue ($n = 5$), as described in Fig. 1. $^*P < 0.001$ (in *A*), $S2 < P1$ and $P2$. $^*P < 0.01$ (in *B*), $S2 > P1$ and $P2$.

trast, ACE activity was higher in the high-detergent membrane fraction (*S2*: 0.339 ± 0.008 μmol·g protein^{-1}·min^{-1}) than in the low-detergent membrane fractions (*P1* and *P2*: 0.022 ± 0.022 and 0.087 ± 0.056 μmol·g protein^{-1}·min^{-1}, $P < 0.01$; Fig. 2*B*). Nevertheless, total ANG II formation from chymase predominated over ANG II formation from ACE not only in low-detergent membrane fractions (*P1* and *P2*: 15.3 ± 3.4 and 13.3 ± 2.9 vs. 0.022 ± 0.022 and 0.087 ± 0.056 μmol·g protein^{-1}·min^{-1}) but also in the high-detergent membrane fraction (*S2*: 0.96 ± 0.21 vs. 0.339 ± 0.008 μmol·g protein^{-1}·min^{-1}).

Species Differences in ANG II Formation

Total intracardiac ANG II formation from ACE and chymase was determined utilizing the HD (*S2*) and LD (*P1*) membrane preparations as follows

$$\text{total ANG II formation } (\mu\text{mol/g/min}) = [\text{chymase activity}]^{P1} + [\text{ACE activity}]^{S2}$$

The percent ANG II formed from ACE was determined from ACE activity in the high-detergent membrane fraction (*S2*) divided by total ANG II formation as defined above, whereas the percent ANG II formed from heart chymase was determined from chymase activity in the low-detergent membrane fraction (*P1*) divided by total ANG II formation as defined above.

Figure 3 demonstrates that total ANG II formation from ACE and chymase, as defined by the equation above, was highly variable across species. Total ANG II formation was greater in human and dog hearts (15.8 ± 3.4 and 3.90 ± 0.35 μmol·g protein^{-1}·min^{-1}, respectively) than in rat, rabbit, and mouse hearts (0.41 ± 0.02, 0.61 ± 0.07, and 1.16 ± 0.08 μmol·g protein^{-1}·min^{-1}, respectively), and this difference was due to greater chymase activity (Fig. 4*A*). In contrast, total ANG II formation from ACE did not differ among human, dog, rat, and rabbit hearts (0.34 ± 0.01, 0.32 ± 0.06, 0.31 ± 0.02, and 0.55 ± 0.06 μmol·g protein^{-1}·min^{-1}, respectively) but was higher in the mouse heart (1.09 ± 0.05 μmol·g protein^{-1}·min^{-1}, $P < 0.001$; Fig. 4*B*). Taken together, percent ANG II formation from chymase predominated over ACE in human (97.5 ± 0.2 vs. 2.5 ± 0.4%) and dog (92.3 ± 3.0 vs. 7.7 ± 2.3%) hearts, whereas ACE predominated over chymase in rat (75.6 ± 1.5 vs. 24.4 ± 0.4%), rabbit (90.2 ± 5.7 vs. 9.8 ± 5.7%), and mouse hearts (94.0 ± 0.6 vs. 6.0 ± 0.6%; Fig. 5).

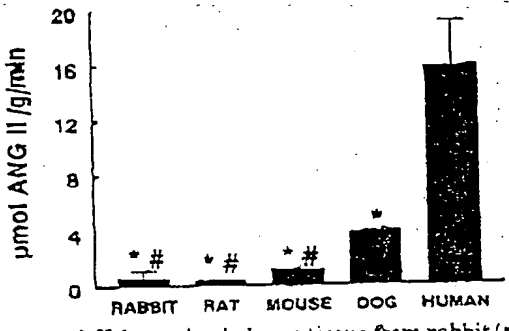

Fig. 3. Total ANG II formation in heart tissue from rabbit ($n = 3$), rat ($n = 8$), mouse ($n = 6$), dog ($n = 6$), and human ($n = 5$). $^*P < 0.05$ vs. human; $^\#P < 0.05$ vs. dog.

DISCUSSION

The major finding of the current study was that ACE activity was 16-fold higher in high- than in low-detergent preparations, whereas chymase activity was 15-fold higher in low- than in high-detergent human cardiac membrane preparations. By use of combined high- and low-detergent membrane preparations, chymase predominated over ACE activity in human and dog hearts, whereas ACE was the predominant ANG II-forming mechanism in mouse, rat, and rabbit hearts. The presence of chymase accounted for the extremely high total ANG II formation in human heart compared with other species. Whether enhanced ANG II formation from chymase in the human heart is of physiological or pathophysiological significance is a source of controversy.

Studies in animals known to express chymase have yielded contradictory results regarding the roles of ACE vs. chymase in ANG II formation. In conscious baboons, intravenous infusion of the chymase-specific substrate [Pro^{11}-D-Ala^{12}]ANG I resulted in increased left ventricular systolic and diastolic pressures consistent with arterial vasoconstriction (6). These effects were antagonized by the ANG II receptor antagonist losartan but not by an ACE inhibitor, thus demonstrating the in vivo contribution of chymase to ANG II generation. In patients with peripheral vascular disease, maximal walking distance and subjective symptoms were improved by nafamostat, a serine protease

they readily cleave the Tyr^4-Ile^5 and Phe^8-His^9 bonds in angiotensins, thus inactivating them. The action of rat chymase-3 could account for the chymostatin-inhibitable ANG II formation in our rat heart assays. This may, in part, explain the failure of ACE inhibitor therapy to attenuate pressure (15, 18, 28) and volume (19) overload-induced left ventricular hypertrophy in the rat, which has been observed in some studies. These findings suggest incomplete inhibition of ANG II formation by ACE inhibitors and/or additional ANG II-forming pathways. In addition, stretch of neonatal rat myocytes has been shown to induce release of ANG II that is independent of an increase in ACE activity and unaffected by prior treatment with ACE inhibitor (20). Taken together, in vivo and in vitro data suggest that there are non-ACE-dependent pathways of ANG II formation in the rat heart that may be attributed to rat chymase-3.

Differences among species in the relative contributions of ACE and chymase to ANG II formation in heart reflect the limitations of extrapolating from animal models of heart failure and cardiac hypertrophy to the human. Use of ACE inhibitors in the routine treatment of heart failure makes this issue even more important, since inhibition of intracardiac ACE with these drugs would increase ANG I levels and shunt this substrate to chymase preferentially. Our results suggest that ANG II-forming capacity in vitro should be assessed using a combined approach that optimizes the conditions for membrane-bound (ACE) and intracellular and interstitial (chymase) location of these enzymes.

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Address for reprint requests: L. J. Dell'Italia, Div. of Cardiology, Dept. of Medicine, University of Alabama at Birmingham, 310 Lyons Harrison Research Bldg., 701 South 19th St., Birmingham, AL 35294.

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Articles

Randomised trial of losartan versus captopril in patients over 65 with heart failure (Evaluation of Losartan in the Elderly Study, ELITE)

*Bertram Pitt, Robert Segal, Felipe A Martinez, Georg Meurers, Alan J Cowley, Ignatius Thomas, Prakash C Deedwania, Dawn E Ney, Duane B Snavely, Paul I Chang, on behalf of ELITE Study Investigators**

Summary

Background To determine whether specific angiotensin II receptor blockade with losartan offers safety and efficacy advantages in the treatment of heart failure over angiotensin-converting-enzyme (ACE) inhibition with captopril, the ELITE study compared losartan with captopril in older heart-failure patients.

Methods We randomly assigned 722 ACE inhibitor naive patients (aged 65 years or more) with New York Heart Association (NYHA) class II–IV heart failure and ejection fractions of 40% or less to double-blind losartan (n=352) titrated to 50 mg once daily or captopril (n=370) titrated to 50 mg three times daily, for 48 weeks. The primary endpoint was the tolerability measure of a persisting increase in serum creatinine of 26·5 μmol/L or more (≥0·3 mg/dL) on therapy; the secondary endpoint was the composite of death and/or hospital admission for heart failure; and other efficacy measures were total mortality, admission for heart failure, NYHA class, and admission for myocardial infarction or unstable angina.

Findings The frequency of persisting increases in serum creatinine was the same in both groups (10·5%). Fewer losartan patients discontinued therapy for adverse experiences (12·2% *vs* 20·8% for captopril, p=0·002). No losartan-treated patients discontinued due to cough compared with 14 in the captopril group. Death and/or hospital admission for heart failure was recorded in 9·4% of the losartan and 13·2% of the captopril patients (risk reduction 32% [95% CI −4% to +55%], p=0·075). This risk reduction was primarily due to a decrease in all-cause mortality (4·8% *vs* 8·7%; risk reduction 46% [95% CI 5–69%], p=0·035). Admissions with heart failure were the same in both groups (5·7%), as was improvement in NYHA functional class from baseline. Admission to hospital for any reason was less frequent with losartan than with captopril treatment (22·2% *vs* 29·7%).

Interpretation In this study of elderly heart-failure patients, treatment with losartan was associated with an unexpected lower mortality than that found with captopril. Although there was no difference in renal dysfunction, losartan was generally better tolerated than captopril and fewer patients discontinued losartan therapy. A further trial, evaluating the effects of losartan and captopril on mortality and morbidity in a larger number of patients with heart failure, is in progress.

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Introduction

Angiotensin-converting-enzyme (ACE) inhibitors reduce morbidity and mortality in patients with chronic heart failure and systolic left-ventricular dysfunction as well as in patients who have had a myocardial infarction.[1–4] The benefits of ACE inhibitors have been mostly attributed to blockade of angiotensin II production and/or to a decrease in the breakdown of bradykinin.[5–11] Bradykinin has been shown to have beneficial effects associated with the release of nitric oxide and prostacyclin, which may contribute to the haemodynamic effects of ACE inhibition. Bradykinin may, however, also be responsible for some of the adverse reactions to ACE inhibitors such as cough, angio-oedema, renal dysfunction, and hypotension,[12–14] and these side-effects may explain in part why ACE inhibitors are used in less than 30% of patients with heart failure despite the proven clinical benefit of these agents.[15]

Orally active, non-peptide angiotensin II type 1 receptor antagonists such as losartan can block this receptor specifically without increasing bradykinin levels,[16] and since angiotensin II may be produced by alternate pathways[17–20] such drugs may have additional advantages over ACE inhibitors where blockade of the effects of angiotensin II is incomplete. Losartan is licenced for the treatment of hypertension in many countries, and in earlier studies in patients with symptomatic heart failure, oral losartan produced beneficial haemodynamic effects both acutely and with chronic dosing.[21,22]

The Evaluation of Losartan in the Elderly (ELITE) study has compared effects on renal function, morbidity/mortality, and tolerability of long-term treatment with losartan or captopril in patients aged 65 years and older with symptomatic heart failure. The primary endpoint was the tolerability measure of a persisting increase in serum creatinine of 0·3 mg/dL (26·5

*Investigators listed at end of paper

Division of Cardiology, University Hospital, Ann Arbor, MI, USA (B Pitt MD); **Instituto de Medicina la Paz, Cordoba, Argentina** (F A Martinez MD); **Innere Medizin-Kardiologie, Krankenhaus Vinzentinum, Ruhpolding, Germany** (G Meurers MD); **Department of Cardiovascular Medicine, Queen's Medical Centre-University Hospital, Nottingham, UK** (A J Cowley DM); **Medical Research Institute, Slidell Memorial Hospital and Medical Center, Slidell, LA, USA** (I Thomas MD); **Cardiology Section, University of California San Francisco, VA Medical Center, Fresno, CA, USA** (P C Deedwania MD); **and Merck Research Laboratories, West Point, PA, USA** (R Segal MD, D E Ney, D B Snavely, P I Chang MD)

Correspondence to: Dr Bertram Pitt, Division of Cardiology, University Hospital, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0368, USA

Panel 1: Enrolment criteria

Inclusion criteria
Age ≥65
NYHA class II, III, or IV and ejection fraction ≤40%
Never received an ACE inhibitor and stable cardiovascular therapy

Clinical exclusion criteria
Systolic BP <90 mm Hg or uncontrolled hypertension (diastolic >95 mm Hg)
Significant obstructive valvular disease or symptomatic ventricular or supraventricular arrhythmia
Constrictive pericarditis or active myocarditis
Cardiac surgery likely during study period or angioplasty within previous 72 h, bypass surgery within 2 wk, or ICD within 2 wk
Acute myocardial infarction in previous 72 h, unstable angina (requiring admission) within 3 mo, or angina (requiring S glyceryl trinitrate tablets/wk) within 6 wk
Stroke or transient ischaemic attack in previous 3 mo
Digitalis toxicity, uncontrolled diabetes, chronic cough of any aetiology, untreated thyrotoxicosis or hypothyroidism, renal-artery stenosis, angio-oedema of any aetiology, haematuria of unknown aetiology
Condition that would contraindicate a vasodilator
Unlikely survival for length of study or risk to patient

Laboratory exclusion criteria
Serum creatinine ≥221 μmol/L (2·5 mg/dL) potassium <3·5 or >5·5 mmol/L, magnesium <0·7 mmol/L
Transaminases >twice upper limit of normal
Haemoglobin <10 g/dL or haematocrit <30%, white blood cell count 3000×10^6/L, or platelets 100×10^9/L

Other exclusion criteria
Another investigational drug in previous 4 wk
Inability to give informed consent
Potentially noncompliant (eg, alcohol or drug abuse)
Previous treatment with losartan or other angiotensin II antagonist

μmol/L) or more on therapy; the secondary endpoint was the composite efficacy measure of death and/or hospital admissions for heart failure. Other prespecified efficacy measures included total mortality and hospital admission for heart failure separately, New York Heart Association (NYHA) functional class, and admission to hospital for myocardial infarction or unstable angina.

Patients and methods

The ELITE study[10] was a prospective double-blind, randomised, parallel, captopril-controlled clinical trial conducted at 125 centres in the United States, Europe and South America. The study was approved by institutional review boards at each site; all patients gave written informed consent. An independent Data and Safety Monitoring Committee monitored the progress of the study.

Patient population

Patients were aged 65 years or more (two-thirds were 70 or older) with symptomatic heart failure (NYHA II–IV), decreased left-ventricular ejection fraction of 40% or less, and no history of prior ACE inhibitor therapy. Enrolment and exclusion criteria are summarised in panel 1.

Randomisation and study therapy

After a 2-week placebo run-in, patients were randomised to 48 weeks of active therapy, either to captopril 6·25 mg titrated to 12·5, 25, and then 50 mg three times daily (plus placebo for losartan) or to losartan 12·5 mg, titrated to 25 and then 50 mg once daily (plus placebo for captopril). Titration generally occurred at 7-day intervals as tolerated. Treatment with all other concomitant cardiovascular therapies was permitted with the exception of open-label ACE inhibitors. Randomised patients were stratified by age (<70, ≥70).

Evaluation of patients

Clinical assessments were done weekly during titration and 3-monthly thereafter. Laboratory evaluations were done at weeks 3, 6, and 12, and every 3 months after that.

Study endpoints

The primary endpoint was a safety measure of renal dysfunction, defined as an increase in serum creatinine by 26·5 μmol/L or more (0·3 mg/dL or more) from baseline (last measurement before randomisation) that was confirmed by a repeat measurement 5–14 days later, during continued treatment. All-cause mortality and hospital admission for heart failure were also prespecified endpoints. The composite of death and/or admission for heart failure was added as the secondary endpoint by protocol amendment on completion of patient participation in the study before unblinding. This change was prompted by data from two placebo-controlled 12-week exercise studies (of about 350 patients each), demonstrating a beneficial effect on this endpoint.[11] The Data and Safety Monitoring Committee was notified in advance. Hypotension-related symptoms, clinically important serum potassium increases (0·5 mmol/L or more), and cough (all originally secondary endpoints) became tertiary endpoints. All deaths (including cause of death) and hospital admissions were adjudicated on by an independent Clinical Endpoint Adjudication Committee, blinded to study treatment (see panel 2 for mortality classification). Other prospectively defined endpoints were admission to hospital for myocardial infarction or unstable angina, worsening of heart failure (NYHA functional classification), withdrawal from the study due to study drug intolerance, and changes in neurohormonal profile.

Statistical methods

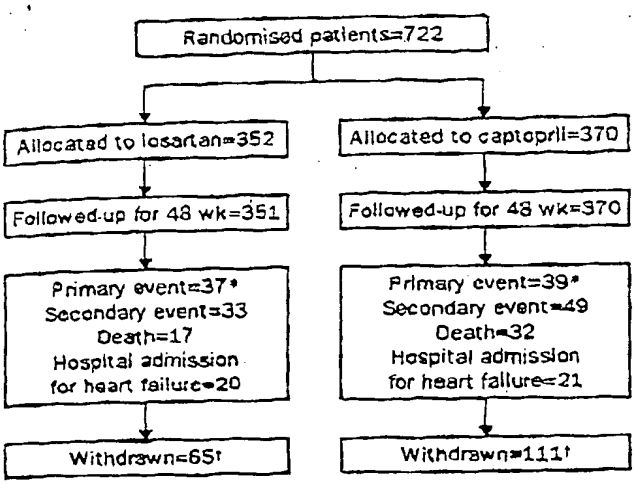
The study was designed[10] with 90% power to detect a 40% reduction in persistent renal dysfunction events, assuming a rate of 30% for such an event in the captopril group. The event rate proved to be only 10·5% for captopril so the power to detect a 40% reduction was substantially decreased. Analysis of increases in serum creatinine was based upon a modified intent-to-treat population—ie, all patients were analysed according to their randomisation group, and an endpoint was declared only if the initial and confirmatory increases in creatinine occurred while the patient was on double-blind therapy. Patients who withdrew from the study without meeting this endpoint were censored in the time-to-event analysis at the time of study discontinuation.

Analyses of deaths and heart-failure admissions (adjudicated endpoints) were based on an intent-to-treat population; all patients who discontinued prematurely were followed up to the specified 48 weeks. Patients not meeting the endpoint were censored in the time-to-event analysis either at the time of study completion for patients who completed or at the end of the 48-week follow-up period for patients who discontinued.

For all time-to-event data, survival analyses were based upon the log-rank test. The effect of treatment group in the model was tested with a control for stratification (age <70 or ≥70). The time to first event was used for each endpoint. Risk reductions were based upon Mantel-Haenszel adjusted (for age category) relative risk estimates.

Panel 2: Mortality classification

Sudden cardiac death—Without warning or within 1 h of symptoms
Death due to progressive heart failure—Preceded by worsening signs and/or symptoms of heart failure, including cardiogenic shock
Fatal myocardial infarction—Necropsy-verified myocardial infarction or death within 28 days of a hospital-verified acute myocardial infarction, provided no other cardiac or non-cardiac cause of death is found
Death due to other cardiac causes—Such as arrhythmia
Death due to other vascular causes—Such as stroke, pulmonary embolus, and ruptured aneurysm
Death due to non-cardiovascular causes—Not due to any cardiac or vascular events



*While on study therapy; † withdrawn from assigned therapy but still followed-up for the intention-to-treat analysis of secondary or secondary endpoint.

Figure 1: **Study profile**

Adverse effects, such as discontinuations for study drug intolerance, cough, and hypotension-related symptoms, were analysed by the Cochran-Mantel Haenszel general association test adjusted for age category. Changes in NYHA functional class were analysed by Wilcoxon rank-sum test.

No adjustments were made for the multiplicity of the secondary or other endpoints; unadjusted p values are presented.

Results

Recruitment began in May, 1994; the last patient was enrolled in July, 1995; and follow-up was completed in June, 1996. Enrolment of patients at the 125 participating centres ranged from 1 to 93 (median of 4); recruitment proved difficult and a substantial number of sites were required to achieve the study sample. Of the 722 patients enrolled, 352 were randomised to losartan and 370 to captopril (figure 1). The two treatment groups were similar with respect to all baseline characteristics (table 1). Concomitant therapies during the study were similar between the two treatment groups; diuretics were used in over 75% of patients, digitalis in over 55% of patients, and non-ACE-inhibitor vasodilatory drugs (including hydralazine and nitrates) in over 40% of patients in both treatment groups. 300 patients (85%) were titrated to the target dose of losartan 50 mg once daily and 310 patients (84%) in the captopril group were titrated to the target dose of 50 mg three times a day. 75% of losartan-treated patients were receiving the targeted dose of 50 mg daily (mean daily dose 42·6 mg for all patients) and 71% of captopril patients were receiving the targeted dose of 50 mg three times a day (mean 122·7 mg).

Renal dysfunction

There was no significant difference between losartan and captopril in the frequency of the primary endpoint—namely, persisting (confirmed) increases in serum creatinine (10·5% in each group, table 2; risk reduction 2% [95% CI −51% to +36%], p=0·63). Single rises in serum creatinine of 26·5 μmol/L or more were documented in 92 losartan-treated patients (26·1%) and 110 captopril-treated patients (29·7%). Of these 202 patients, 68% (137) had confirmation measurements done while on active therapy per protocol, and 55% (76) of those with confirmation measurements met the endpoint.

Characteristic	Losartan (n=352)	Captopril (n=370)
Demographic		
M/F	234/118	248/122
Age (<70/>70)	95/257	119/251
Mean age (yr)	74 (5·8)	73 (6·1)
Race (white/black/other)	320/16/16	326/18/26
Current cigarette smokers	39	45
Clinical		
Heart failure* due to ischaemic/non-ischaemic heart disease	242/110	250/120
NYHA class II/III/IV	231/116/5	237/126/7
Drug therapy		
Diuretics	280	275
Digitalis	199	209
Hydralazine	12	12
Nitrates	180	191
Calcium channel blockers	123	121
Potassium	91	89
Anticoagulants	60	68
Aspirin	178	166
β-blockers	55	63
Antiarrhythmics	37	39
Secondary diagnoses*		
Myocardial infarction	184	177
Hypertension	201	212
Atrial fibrillation	86	82
Diabetes mellitus	84	89
Renal insufficiency	21	26
Stroke	32	37
Measurements		
Ejection fraction (%)	31 (7·2)	30 (7·6)
Serum creatinine (μmol/L)	106 (35)	106 (35)
Serum potassium (mmol/L)	4·3 (0·4)	4·3 (0·5)
Heart rate (b/min)	73 (11·7)	74 (10·4)
Systolic BP (mm Hg)	137 (17·6)	137 (19·1)
Diastolic BP (mm Hg)	79 (9·4)	79 (10·6)
Weight (kg)	76 (33·1)	74 (33·8)

*Based on patient history.
Table shows numbers or, for age and for measurements, mean (SD).

Table 1: **Baseline clinical characteristics and drug therapy**

Death and/or heart failure admission

Follow-up data on death and hospital admission were complete except for one losartan-treated patient who discontinued after one dose. During the course of the study, death and/or heart failure admissions occurred in 33 of 352 losartan-treated patients (9·4%) and 49 of 370 captopril-treated patients (13·2%). The risk reduction was 32% (95% CI −4% to +55%, p=0·075; table 3). This risk reduction was primarily due to a decrease in all-cause mortality (4·8% *vs* 8·7%; risk reduction 46% [95% CI 5–69%, p=0·035 (table 3). The cumulative survival curves (intent-to-treat) separated early and remained separated throughout the 48 weeks (figure 2). Analyses by cause (table 3) suggests that, though the numbers of events are small, the lower total mortality in the losartan group is primarily due to a reduction in sudden cardiac deaths. The mortality difference was generally consistent across different subgroups—namely, age, ejection fraction, aetiology of heart failure, NYHA functional class, and concomitant digoxin use, the exception being deaths in women (9/118 losartan *vs* 8/122 captopril).

Age	Treatment	No	Event rate*
All patients	Losartan	352	37 (10·5%)
	Captopril	370	38 (10·5%)
<70	Losartan	95	8 (8·4%)
	Captopril	119	10 (8·4%)
≥70	Losartan	257	29 (11·3%)
	Captopril	251	29 (11·6%)

*Defined as a rise of 26·5 μmol/L (0·3 mg/dL) or more, confirmed within 5–14 days.

Table 2: **Frequency of increases in serum creatinine**

Endpoint	Losartan (n=352)	Captopril (n=370)	Risk reduction (CI)*	p†
Combined death and/or hospital admission for heart failure	33 (9·4%)	49 (13·2%)	0·32 (−0·04 to +0·55)	0·075
All deaths	17 (4·8%)	32 (8·7%)	0·46 (0·05–0·69)	0·035
Cardiovascular	5 (1·4%)	14 (3·8%)	0·64 (0·03–0·86)	
Sudden death	1	1	−0·11 (−20·23 to +0·84)	
Progressive heart failure	1	4 (1·1%)	0·78 (−0·83 to +0·97)	
Myocardial infarction	5 (1·4%)	5 (1·4%)	−0·03 (−2·63 to +0·71)	
Other vascular	5 (1·4%)	8 (2·2%)	0·35 (−0·94 to +0·78)	
Non-cardiovascular				
Hospital admissions	20 (5·7%)	21 (5·7%)	0·04 (−0·74 to +0·47)	0·89
For heart failure	78 (22·2%)	110 (29·7%)	0·26 (0·05–0·43)	0·014
For any reason				

*Reduced risk of endpoint on losartan compared with captopril (negative number denotes increase in risk); estimates control for age category; CI=95% confidence interval.
†Log-rank test (survival analysis) with age category included as stratification factor.

Table 3: **Deaths (and causes of death) and admissions for heart failure or for any reason**

Fewer losartan-treated patients were admitted to hospital for any reason during the 48 weeks but frequency of admission because of heart failure was identical (5·7%) (table 3).

NYHA class

NYHA functional class improved similarly with losartan and captopril treatment ($p \leq 0·001$ versus baseline for both groups); 80% of losartan-treated patients and 81% of captopril-treated patients were class I or II at the end of the study compared with 66% and 64%, respectively, at baseline.

Plasma norepinephrine

A 3% decrease from a geometric mean at baseline of 2·78 μmol/L (469 pg/mL) was observed at 48 weeks with losartan treatment, as compared with a 5% increase from 2·51 μmol/L (424 pg/mL) with captopril ($p=0·49$).

Safety, tolerability and discontinuations

65 (18·5%) of the losartan-treated patients discontinued study therapy or died compared with 111 (30%) captopril-treated patients ($p \leq 0·001$) and the reasons are listed in table 4. Excluding deaths, 43 (12·2%) losartan-treated patients discontinued for adverse effects versus 77 (20·8%) captopril-treated patients. Discontinuations due to worsening heart failure occurred in nine captopril-treated patients and three losartan-treated patients. 14 captopril-treated patients but none of the patients on losartan discontinued study therapy due to cough.

The differential discontinuation rate did not account for the 46% difference in total mortality in the intent-to-treat analysis because this difference was observed predominantly in patients who remained on study therapy. Per protocol analysis gave death rates of 3·7% (11/298) for for losartan and 8·5% (24/282) for captopril (risk reduction 57% [95% CI 13–78%], $p=0·013$).

There were no differences between the two treatment groups with respect to hypotension-related symptoms (occurring overall in 24% of patients). First-dose hypotension was reported in seven captopril and four losartan treated patients. Persisting increases in serum potassium of 0·5 mmol/L or more above baseline while on therapy was observed in 66/352 (18·8%) losartan-treated and 84/370 (22·7%) captopril treated patients ($p=0·069$).

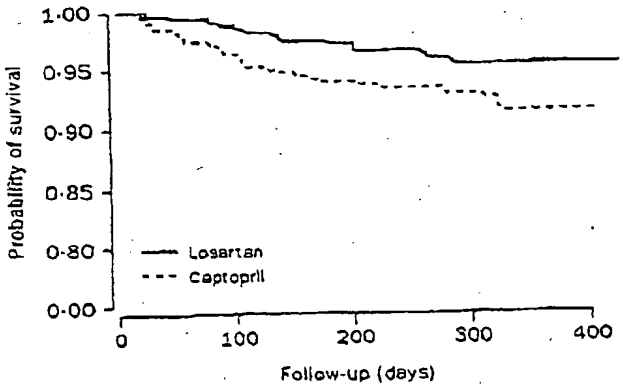


Figure 2: **Kaplan-Meier survival curves among patients with chronic heart failure in losartan and captopril groups**
Patients in losartan group had a 46% lower risk of death than patients in captopril group ($p=0·035$). Patients were followed up for 48 weeks.

Discussion

ELITE is the first long-term (48 weeks) study comparing an angiotensin II type 1 receptor antagonist with an ACE inhibitor in patients with symptomatic heart failure and systolic left-ventricular dysfunction. Captopril was chosen as the comparison ACE inhibitor drug because it may have fewer renal effects than longer-acting ACE inhibitors.[9] The incidence of persistent renal dysfunction was not different between the losartan and captopril groups (both 10·5%), and fewer than 2% of patients discontinued for this reason in either group.

Reason	Losartan (n=352)	Captopril (n=370)
All	65 (18·5%)*	111 (30·0%)
Adverse event (excluding death)†	43 (12·2%)*	77 (20·8%)
Cough†	0*	14 (3·8%)
Worsening heart failure	3	9
Hyperkalaemia†	2	6
Taste perversion/ appetite decreased	0	8
Rash	0	4
Hypotension†	8	5
Angio-oedema	0	3
Arrhythmia	1	4
Myocardial infarction	3	4
Renal dysfunction†	5	3
Stroke	4	3
Angina/unstable angina	3	2
Other non-cardiac cause	14	14
Death	1	5
Therapy ineffective	0	1
Protocol deviation	5	7
Patient withdrew	16	19
Other	0	2

*$p<0·002$. †These individual adverse experiences causing discontinuation were tested for significance (according to protocol) and are based on reasons cited by investigator.

Table 4: **Primary reason for discontinuation of study medication before completion of study**

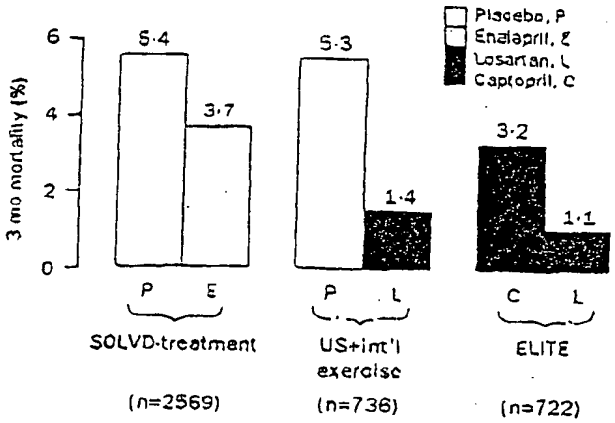


Figure 3: **3-monthly mortality in SOLVD-treatment, exercise studies, and ELITE**

Discontinuation due to other adverse effects was significantly more common in patients randomised to captopril. 27 patients withdrew from the captopril group because of altered taste sensation, rashes, or angio-oedema, all side-effects recognised with ACE inhibitors.[12,14] None of the losartan-treated patients stopped the drug for any of these reasons and the overall tolerability of losartan was superior to that to captopril.

Although no significant difference was observed for the primary endpoint (persistent renal dysfunction) or the secondary endpoint (composite of death/heart failure admissions), treatment with losartan was associated with less all-cause mortality than captopril, a drug known to have survival benefits.[5,7,8] The survival benefit of losartan was observed early in the study, persisted throughout the 48 weeks, and was consistent among all subgroups except female patients. Only one-third of the patients enrolled in ELITE were women, and mortality in women with heart failure taking losartan or captopril requires further study. The greater drop-out rate in patients on captopril did not account for the beneficial effects of losartan on total mortality; the difference in total mortality was primarily observed in those who remained on active therapy.

An improvement in survival with losartan of similar magnitude has been observed in two placebo-controlled 12-week exercise studies in about 350 patients with symptomatic heart failure.[15] Losartan did not improve treadmill exercise duration (the primary endpoint) but was associated with an unexpected reduction in mortality. Mortality rates in the placebo controls in the exercise studies and the captopril controls in ELITE are comparable with the rates for the placebo and enalapril groups, respectively, in the SOLVD trial[2] (figure 3). Apart from age (ELITE patients were older), the patient populations in these studies are similar. The limitations of cross-study comparisons and the greater age of the patients in this study notwithstanding, treatment with losartan in the exercise studies and in ELITE was associated with comparably low mortality rates which were less than the observed mortality rates for both placebo and enalapril in SOLVD.

Before the availability of ACE inhibitors, progressive heart failure accounted for about 50% of deaths in patients with heart failure.[16] In patients with mild-to-moderate heart failure who are treated with an ACE inhibitor, death due to progressive heart failure is much less common and sudden cardiac death is now the predominant category. For example, in the discontinued SWORD trial—in which patients with mild-to-moderate heart failure or left-ventricular dysfunction were randomised to d-sotalol or placebo on a background of usual therapy, including an ACE inhibitor—arrhythmia accounted for about two-thirds of total mortality in the placebo group while progressive heart failure accounted for only one-sixth.[17] In ELITE sudden cardiac death was the most common cause of death in the captopril patients, and the apparent mortality advantage for losartan seems primarily to be due to a reduction in sudden cardiac death. Few patients died due to progressive heart failure or to fatal myocardial infarction in either group in ELITE.

If it is confirmed that losartan does reduce the risk of sudden cardiac death it will be necessary to find out if this property is specific to this agent or is a class effect of angiotensin II type I receptor antagonists and due to the more complete blockade of angiotensin II that these antagonist drugs achieve. ACE activity may not be completely suppressed by captopril. However, the captopril regimen in ELITE (target dose 50 mg three times daily) is one that has been found effective in several studies in heart failure,[5,7,8] and considered to have mortality benefits.[7,16] Angiotensin II may also be formed by non-ACE-dependent pathways,[17,18] and the more complete blockade of angiotensin II effects by losartan may result in more complete suppression of catecholamines at the tissue level.[19] Furthermore, bradykinin, which releases norepinephrine,[20] is not increased with direct angiotensin II blockade[21] while it is with ACE inhibitors.

In this study NYHA functional class improved significantly and to a comparable extent from baseline after long-term treatment with both losartan and captopril, and rates of hospital admission for heart failure were similar too. Another similarity was the low incidence of death due to progressive heart failure (less than 1% for both losartan and captopril).

This study was in patients aged 65 years and older with systolic left-ventricular dysfunction, and the results cannot be extrapolated to younger heart-failure patients or patients with diastolic dysfunction. Elderly patients were chosen because most patients with heart failure are over 65. Elderly patients tend to be under-represented in randomised trials in heart failure (eg, in SOLVD the mean age was about 61 years compared with 73·5 in ELITE).

We chose an active drug as the control arm in ELITE so that no patient would be denied any benefit of renin-angiotensin system blockade and we selected captopril because there may be less renal dysfunction (the primary endpoint in ELITE) with this shorter-acting agent than with a longer-acting ACE inhibitor.[8] Captopril had been shown to be effective in heart failure and left-ventricular systolic dysfunction,[5,7,8] and a meta-analysis suggests that the reduction in mortality is consistent among the various ACE inhibitors in a broad range of patients with heart failure.[22] This study demonstrated that losartan reduced mortality compared with captopril; whether the apparent mortality advantage for losartan over captopril holds true for other ACE inhibitors requires further study.

Structure of ELITE Study Group

Data and safety monitoring board—C Furberg (chair), J Neaton, J Poole-Wilson, G Rieger, J Wei.

Clinical endpoint adjudication committee—J Burke, L Prame, H Levine, E Loh.

Neurohormone analyses—C Hall.

Central laboratories—Corning SciCor, Inc.

Principal investigator—B Pitt.

Members of ELITE Study Group—USA: *Albany, NY*: R Cuppett; *Ann Arbor, MI*: J Bengston and J Nicklas; *Atlanta, GA*: D Schroeder; *Aurora IL*: M Marzec; *Baltimore, MD*: D Goldscher and S Sneed; *Beverly Hills, CA*: R Karlsberg; *Birmingham, AL*: V Bittner; *Bronx, NY*: N Bhalodkar; *Buffalo, NY*: R Kuhn; *Charleston, SC*: G Hendrix; *Clearwater, FL*: K Kaplan; *Columbus, OH*: R Cody; *Dallas, TX*: M Berk; *Dearborn, MI*: A Riba; *Easton, PA*: T Little; *Elgin, IL*: A Nisar; *Encinitas, CA*: A Saleh; *Eugene, OR*: K Jacobson; *Fairhope, AL*: S Self; *Fort Myers, FL*: J Conrad; *Framingham, MA*: E Hoffer; *Fresno, CA*: P Deedwania; *Huntingdon, WV*: R Touchon; *LaJolla, CA*: A Johnson; *Lakeland, FL*: H Narvarte; *LaMesa, CA*: L Sprinkle; *Larkspur, CA*: J Sklar; *Lexington, KY*: A Cross; *Los Angeles, CA*: B Singh; *Madison, WI*: J Farnham and C Vander Ark; *Memphis, TN*: M Awdeh; *Miami Beach, FL*: R Scheib; *Montgomery, AL*: P Moore; *New Haven, CT*: L Deckelbaum and T Ramahi; *New Orleans, LA*: C A Deabate and W Smith; *Newport News, VA*: L McLaurin; *Oklahoma City, OK*: C Corder; *Omaha, NE*: D Chapman; *Orange, CA*: D Abrahamson and W Johnston; *Passaic, NJ*: M Schwarz; *Pawtucket, RI*: K LaBresh; *Philadelphia, PA*: S Brozena and I Pina; *Phoenix, AZ*: P Cardello and L K Smith; *Pittsburgh, PA*: A Gradman; *Providence RI*: S Sharma; *Renton, WA*: J Nemanich; *Richmond, VA*: P Mohanty; *Rochester, NY*: P Pande; *Sacramento, CA*: W Lewis; *St Louis, MO*: M Rich; *San Diego, CA*: H Ingersoll, D Morth, and H Rockman; *San Francisco, CA*: M Cheitlin; *Shreveport, LA*: J Ghali; *Slidell, LA*: I Thomas; *Springfield, MO*: S Turner; *Staten Island, NY*: D Bloomfield; *Tallassee, AL*: P Bianchi; *Tucson, AZ*: S Goldman; *West Roxbury, MA*: R Shannon; *Westwood, NJ*: J Landzberg; *Wichita, KS*: D Vine; *Winston-Salem, NC*: D Klemm; *Zanesville, OH*: D Myers. International: *Alicante, Spain*: F Sogorb-Garri; *Athens, Greece*: L Anthopoulos and D Kokkinos; *Belfast, UK*: D Johnston; *Bobigny, France*: J Artigou; *Brescia, Italy*: C Alicandri; *Brighton, UK*: S Holmberg; *Brussels, Belgium*: M Telerman; *Cordoba, Argentina*: F Martinez; *Cuenca, Spain*: A Melero-Pita; *Danderyd, Sweden*: N Rehnquist; *Edinburgh, UK*: J McMurray; *Freiburg, Germany*: G Hassenfuss; *Glasgow, UK*: H Daegle and J Reid; *Graz, Austria*: W Klein; *Gunzenhausen, Germany*: F Freytag; *Johannesburg, S Africa*: S Middlemost; *Karlshad, Germany*: C Diehm; *Leeds, UK*: S Ball; *Leon, Spain*: M Rodriguez; *Lima, Peru*: G Rivara, M Sanchez-Palacios, and L Segura; *London, UK*: A Lant; *Lugano, Switzerland*: T Moccetti; *Madrid, Spain*: L Alonso-Pulpon; *Malmo, Sweden*: R Willenheimer; *Manchester, UK*: R Levey; *Mendoza, Argentina*: the late Julio Yusky; *Mexico City, Mexico*: J Rivera; *Moscow, Russia*: V Metelitsa; *Newport, UK*: A Baksi; *Nottingham, UK*: A Cowley; *Ontario, Canada*: M Arnold; *Osnabrück, Germany*: M Hüdepohl; *Petach Tikva, Israel*: R Pas; *Porto, Portugal*: F Rocha-Gonçalves; *Quebec, Canada*: G Houde; *Rehovot, Israel*: A Caspi; *Rome, Italy*: A Maseri; *Romford, UK*: J Stephens; *Ruhpolding, Germany*; G Meurers; *Saint-Michel, France*: D Flammang; *San Luis Potosi, Mexico*: J Arenas; *Santafe de Bogota, Colombia*: J Leon; *Santiago, Chile*: H Prat; *São Paulo, Brazil*: J Ramires; *Valencia, Spain*: F Algarra-Vidal; *Caracas, Venezuela*: E Morales Briceño; *Verwoerdburg, S Africa*: D Myburgh; *Wellington, N Zealand*: N Lever, *Wilrzburg, Germany*: U Nellessen.

Coordinating centre, Merck Research Laboratories—R Segal, P Chang (study directors), D Ney, K Gresh, A Sweet, M VelMs (programme coordinators), D Snavely, D Sharma (statisticians), D Wolf, S Duffy, B Conti, K Rombola, D McLaughlin, J Sickel, R Tierney (data coordination), K Olivera (clinical supplies).

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Contribution of Cardiac Renin-Angiotensin System to Ventricular Remodelling in Myocardial-Infarcted Rats

Hiroyuki Yamagishi, Shokei Kim*, Toshio Nishikimi, Kazuhide Takeuchi and Tadanao Takeda

*First Department of Internal Medicine, and *Department of Pharmacology, Osaka City University Medical School, Abeno-ku, Osaka 545, Japan*

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H. Yamagishi, S. Kim, T. Nishikimi, K. Takeuchi and T. Takeda. Contribution of Cardiac Renin-Angiotensin System to Ventricular Remodelling in Myocardial-Infarcted Rats. *Journal of Molecular and Cellular Cardiology* (1993) 25, 1369–1380. To investigate the contribution of the cardiac renin-angiotensin system to ventricular dilatation after myocardial infarction, we examined the effects of 3-week treatments with an angiotensin converting enzyme inhibitor, delapril, and a selective angiotensin II type 1 (AT_1) receptor antagonist, TCV-116, on haemodynamics and ventricular angiotensin II contents in myocardial-infarcted rats. TCV-116 reduced mean aortic pressure, and prevented the increase of right and left ventricular weight, left ventricular end-diastolic pressure and volume of myocardial-infarcted rats, to a similar extent to delapril. Thus, AT_1 receptor-mediated action of angiotensin II plays a central role in the development of ventricular dilatation. Angiotensin II contents in the right and non-infarcted left ventricles (6.0 ± 1.0 and 5.9 ± 0.7 pg/g tissue, respectively, mean ± S.E.M.) of myocardial-infarcted rats were not different from those of sham-operated rats. However, angiotensin II contents in the infarcted scar (21.7 ± 3.5 pg/g) of myocardial-infarcted rats were 4.2-fold higher than those in the left ventricle of sham-operated rats. Delapril reduced angiotensin II contents in the right and non-infarcted left ventricles, and the scar by 48, 81 and 60%, respectively, but did not reduce plasma angiotensin II in myocardial-infarcted rats. TCV-116 also decreased angiotensin II in the right and non-infarcted left ventricles by 57 and 56%, respectively, while increased plasma angiotensin II by 4.3-fold. Thus, the prevention of ventricular dilatation by these two agents was associated with the decrease in ventricular angiotensin II contents. These observations suggest that the cardiac renin-angiotensin system rather than the circulating system may play an important role in ventricular dilatation after myocardial infarction.

Key Words: Myocardial infarction; Ventricular remodelling; Blockade of AT_1 receptor; ACE inhibition; Cardiac angiotensin II.

Introduction

The progressive left ventricular dilatation after myocardial infarction has been reported (Lamas and Pfeffer, 1991; Pfeffer and Braunwald, 1990). Early after infarction, left ventricular enlargement can occur as a result of infarct expansion, which increases the surface area of the infarcted region by thinning, stretching and outward bulging. During this time, the non-infarcted myocardium also undergoes hypertrophy and lengthening, which leads to increase of the left ventricular cavity radius. The increased left ventricular volume is the most important predictor of subsequent mortality after myocardial infarction (White *et al.*, 1987). This process of the left ventricular dilatation after myocardial infarction has been called ventricular remodelling.

Multiple lines of evidence indicate that angiotensin converting enzyme (ACE) inhibitors and angiotensin (ANG) II type 1 (AT_1) receptor antagonist have favourable effects on ventricular dilatation, heart failure and survival after myocardial infarction (Gay, 1990; Lamas and Pfeffer, 1991; Pfeffer *et al.*, 1985; Raya *et al.*, 1991; Sharpe *et al.*, 1991). Ang II is known to stimulate expression of protooncogene (Hoh *et al.*, 1990), cell growth, and protein synthesis in cultured myocytes (Aceto

Please address all correspondence to: Hiroyuki Yamagishi, First Department of Internal Medicine, Osaka City University Medical School, 1-5-7, Asahi-machi, Abeno-ku, Osaka 545, Japan.

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and Baker, 1990; Baker and Aceto, 1990), and to have cardiotoxic effects when chronically infused to rats (Tan *et al.*, 1991). Thus, the renin-angiotensin system may contribute to the development of ventricular dilatation and heart failure.

Recent observations demonstrated the existence of cardiac renin-angiotensin system, which might play an important role in pathophysiology of cardiovascular diseases (Dzau 1987; Lindpainter and Ganten, 1991). Angiotensinogen mRNA expression is increased in the atria and ventricles of rats in the early phase after experimental myocardial infarction (Drexler *et al.*, 1989), and ACE activity and its mRNA levels are increased in the non-infarcted myocardium of myocardial-infarcted rat (MI rat) (Hirsch *et al.*, 1991). However, no information is available concerning cardiac Ang II contents in MI rats. It is unknown whether the increase in angiotensinogen or ACE gene expression in MI rats leads to the enhanced Ang II synthesis, as the ACE- independent Ang II-forming pathway, namely the alternate pathway, may significantly participate in cardiac Ang II levels (Urata *et al.*, 1991a,b). Thus, it is an open question whether or not cardiac renin-angiotensin system indeed contributes to the pathophysiology of ventricular dilatation and heart failure.

In the present study, we determined cardiac true Ang II contents in MI rats, and investigated the effects of ACE inhibitor, delapril and a newly developed selective, non-peptide AT_1 receptor antagonist, TCV-116 on ventricular Ang II contents.

Materials and Methods

A non-peptide AT_1 receptor antagonist, TCV-116 (Shibouta *et al.*, 1992; Wada *et al.*, 1992) and a non-sulphhydryl ACE inhibitor, delapril hydrochloride (Inada *et al.*, 1986a,b) were donated by Takeda Chemical Industries, Ltd. (Osaka, Japan). TCV-116 at a single oral dose of more than 0.03 mg/kg inhibits the pressor response to Ang II (100 ng/kg, i.v.) over 24 h in conscious rats (Shibouta *et al.*, 1992). In spontaneously hypertensive rats, a single oral administration of TCV-116 at the dose of 1 mg/kg lowers blood pressure by about 45 mm Hg over 24 h (Wada *et al.*, 1992). Thus, TCV-116 is a highly potent and long-acting specific AT_1 receptor antagonist.

Eight-week-old male Wistar rats (purchased from Clea Japan, Inc., Osaka, Japan), weighing 220–270 g, were used in the present study.

Production of myocardial infarction

Myocardial infarction was produced in rats according to a slight modification of the method published by several laboratories (Fishbein *et al.*, 1978; Gay, 1990; Hirsch *et al.*, 1991; Raya *et al.*, 1991). In brief, rats were anaesthetized by intraperitoneal injection of pentobarbital sodium and after intratracheal intubation a left thoracotomy was performed under volume-controlled mechanical ventilation. The heart was expressed from the thorax and a ligature with 6-0 prolene suture was placed around the proximal left anterior descending coronary artery. The chest was then closed. The same surgical procedures to produce infarction were also used in sham-operated rats, except that the suture around the coronary artery was not tied.

Treatment of rats with delapril and TCV-116

Both sham-operated rats and MI rats, which survived 24 h after the surgical operation, were randomly separated into three groups, including untreated, delapril-treated and TCV-116-treated rats. Rats in the delapril-treated group were freely given water containing 1 g/l of delapril. Water intake was approximately 30 ml/rat/day, resulting in a delapril intake of approximately 100 mg/kg/day. Rats in the TCV-116-treated group were administrated 1 mg/kg of TCV-116 once a day by gastric gavage. The TCV-116-treated and untreated rats received normal tap water to drink. All rats were given standard rat chow containing 0.3% Na (CE-2; Clea Japan Inc., Osaka, Japan) and water *ad libitum*. After 3 weeks of treatment, they were studied. Hemodynamic studies were performed on untreated, delapril-treated and TCV-116-treated groups in both sham-operated rats and MI rats. Biochemical studies, including the measurements of plasma renin-angiotensin system and of cardiac ventricular Ang II contents, were performed on untreated sham-

operated rats and untreated, delapril-treated and TCV-116-treated MI rats.

Haemodynamic studies

After 3 weeks of treatment, rats were anaesthetized by intraperitoneal injection of pentobarbital sodium (35 mg/kg body weight). TCV-116-treated rats were studied 12–16 h after the last oral administration of the agent. Body temperature was maintained around 37°C on a heating pad. Aortic and left ventricular pressure were recorded by inserting a polyethylene tubing (0.58 mm internal diameter, PE-50) into the right carotid artery. The catheter was advanced into the aorta and then into the left ventricle. Central venous pressure was measured by cannulating the right external jugular vein with a PE-50 tubing, which was advanced to the region of the thoracic vena cava. Water-filled catheters were connected to a polyethylene tubing (0.86 mm internal diameter, PE-90) connected to a water-filled pressure transducer (model P23 ID, Gould Inc., California, USA). With rats breathing spontaneously, pressures were recorded on a physiological recorder (Polygraph MIC-9800 and Thermal Recorder RF-85, Fukuda Denshi, Tokyo, Japan). Aortic and central venous pressures were recorded as the mean values determined by electronic averaging, and left ventricular systolic pressure and left ventricular end-diastolic pressure were obtained by averaging the values of 10 beats, respectively. Heart rate was determined from the tracing of aortic pressure.

Left ventricular end-diastolic volume

Left ventricular end-diastolic volume was derived from the *ex vivo* left ventricular pressure-volume curve (Gay, 1990; Raya *et al.*, 1991). After completion of the haemodynamic studies, KCl was injected through the jugular venous catheter to arrest the heart in diastole. The heart was rapidly removed with 10 mm of aorta. Two PE-50 catheters, attached to a pressure transducer and an infusion pump (model STC-525, Terumo, Tokyo, Japan), respectively, were inserted into the aortic remnant and then advanced into the left ventricle. The ventricle was isolated by ligation on atrioventricular groove and the right ventricle was incised to prevent any loading effect. After gentle aspiration of the left ventricular cavity to remove any residual blood and to reduce the pressure of −5 mm Hg, saline was infused at the constant rate of 0.68 ml/min into the suspended left ventricle and pressures were simultaneously recorded. When the pressure increased to 30 mm Hg, the infusion was stopped. Three curves were obtained from each left ventricle within 10 min of cardiac arrest. The left ventricular end-diastolic volume was defined as the volume on the recorded pressure-volume curve at a pressure equal to end-diastolic pressure obtained in the haemodynamic studies.

Weighing of right and left ventricles

After the recording of the pressure-volume curves, the right and left ventricles were separated from the atria and the left ventricle was opened with an incision along the septum from base to apex. Both the ventricles were rinsed in normal saline, blotted dry and immediately weighed.

Determination of infarct size

Myocardial infarct size was measured as previously described (Gay, 1990). Three or four longitudinal incisions were made in the septum and infero-posterior wall of left ventricle so that left ventricular tissue could be pressed flat. The circumferences of the left ventricle and the region of transmural infarction, which was recognized as whity scarred tissue, were outlined on two pieces of semi-transparent paper for both the endocardial and epicardial surface. Infarct size was calculated and expressed as a percent of left ventricular surface area, based on the weight of paper of outlined areas. An average of endocardial and epicardial surface areas was reported. Rats with less than 20% of infarct size were excluded from analysis.

Biochemical study

After 3 weeks of treatment, animals were killed by decapitation without prior anesthesia. TCV-116-treated rats were killed 12–16 h after the last oral administration of the agent. For determination of plasma angiotensinogen, renin, Ang II and aldosterone concentrations, trunk blood was collected into pre-chilled

tubes which contained EDTA (1 mg/ml blood) and aprotinin (500 kallikrein inhibitor units/ml blood), which was added to prevent the degradation of Ang peptides in plasma. The plasma was separated by centrifugation at 3000 rpm for 15 min at 4°C, and then 1.5 ml of each sample was immediately applied to the Sep-Pak plus C18 cartridges (Waters, Milford, Mass) as described below.

The heart with 10 mm of aorta was removed rapidly. Catheter was inserted into the aorta and ligated. The heart was perfused with 30 ml of ice-cold saline containing heparin Na (2000 U/l) for 1 min to remove the contaminated blood. The atria and aorta were removed and the right ventricle was incised. After the determination of infarct size as described above, the left ventricle was divided into the infarcted scar tissue and the non-infarcted left ventricle including the interventricular septum. All tissues were blotted dry, weighed and immediately frozen in liquid nitrogen. These procedures were completed within 7 min after the decapitation. Plasma and tissues were stored at −80°C until biochemical studies.

Extraction of ventricles

Extraction of ventricles, for determination of Ang II contents, was performed according to the method of Kim *et al.* (1991c, 1992). Each ventricular tissue was boiled for 5 min in 10 volumes of distilled water, then homogenized in 0.05 N HCl solution. The supernatant, obtained by centrifugation at 15000 rpm for 30 min at 4°C, was immediately applied to a Sep-Pak plus C18 cartridges, as described below.

Measurement of plasma and ventricular Ang II

Measurement of Ang II was carried out by reverse phase high performance liquid chromatography (HPLC) combined with specific radioimmunoassays (RIA), with a slight modification of the previous method (Kim *et al.*, 1991c, 1992). Sep-Pak plus C18 cartridges were pretreated consecutively with 10 ml of methanol, 5 ml of tetrahydrofuran, 5 ml of hexane, 10 ml of methanol and 10 ml of 0.1% trifluoroacetic acid (TFA). The samples of plasma (1.5 ml) and ventricular extracts were applied to the cartridges. Then the cartridges were washed with 10 ml of 0.1% TFA and then 10 ml of the mixture of methanol/water/TFA (10/89.9/0.1, vol/vol/vol). The peptides, retained by the cartridges, were eluted with 3.5 ml of the mixture of methanol/water/TFA (80/19.9/0.1, vol/vol/vol). The eluates were dried in a vacuum centrifuge evaporator (CC-180, Tomy Seiko, Japan) and dissolved in 0.3 ml of 10 mM phosphoric acid/pH 3.8, and chromatographed on an ODS-80TM reverse phase HPLC column (0.46 × 25 cm, Tosoh, Japan). Ang peptides were separated by using a linear gradient of methanol concentration from 30 to 90% in 10 mM phosphoric acid/pH 3.8 for 20 min at the flow rate of 1.0 ml/min. Fractions of 0.4 ml were collected in silicon-coated polypropylene tubes and dried in a vacuum centrifuge evaporator. Each fractionated sample was dissolved in RIA buffer (70 mM sodium phosphate/pH 7.2/50 mM NaCl/2 mM EDTA/0.1 mM diisopropylfluorophosphate/0.3% bovine serum albumin) and was subjected to specific RIA of Ang II. Retention times of Ang I, Ang II, Ang III, Ang (3–8) hexapeptide, Ang (4–8) pentapeptide and Ang (5–8) tetrapeptide were determined by application of synthetic Ang peptides (Peptide Institute, Inc., Osaka, Japan). Specific anti-Ang II serum was raised in rabbits, whose method will be reported in detail elsewhere. The cross-reactivity of anti-Ang II serum used in the present study were: Ang III, 100%; Ang (3–8) hexapeptide, 100%; Ang (4–8) pentapeptide, 100%; Ang (5–8) tetrapeptide, 0%; Ang I, 0.2%. The sensitivity of RIA of Ang II was 0.15 pg/tube. The recovery of ^{125}I-labelled Ang II (1×10^4 cpm), added to plasma, and right and left ventricles, was 68 ± 5 ($n = 4$), 67 ± 7 ($n = 4$) and 64 ± 5% ($n = 4$), respectively.

Measurement of plasma renin concentration

The plasma renin concentration was measured as the rate of generation of Ang I from rat angiotensinogen at 37°C, as previously reported (Kim *et al.*, 1989). Plasma samples were incubated with 24 h-nephrectomized rat plasma as the substrate at 37°C. The generated Ang I was measured by RIA.

Measurement of plasma angiotensinogen

Angiotensinogen was indirectly measured by incubating plasma samples with an excess of pure rat renal renin (50 ng/ml) (Kim *et al.*,

1991a) as previously described (Kim *et al.*, 1991b). The generated Ang I was measured by RIA. Measurement of plasma aldosterone.

The plasma aldosterone concentration was measured by RIA, using a commercially available kit (Sorin Biomedica S.p.A, Italy).

Statistics

Values were expressed as mean ± S.E.M. Statistical comparisons were made with Duncan's method for multiple comparisons after an analysis of variance demonstrated significant differences. Significance level was taken at $P<0.05$.

Results

Haemodynamic studies

Effects of delapril and TCV-116 on ventricular weights and haemodynamic parameters in sham-operated rats

As shown in Table 1, left ventricular weight of sham-operated rats was decreased by 15% ($P<0.01$) by 3 weeks of treatment with delapril, but not by TCV-116 treatment.

As indicated by Figure 1, delapril and TCV-116 lowered mean aortic pressure (126 ± 4, untreated; 104 ± 3, delapril-treated; and 108 ± 4 mm Hg, TCV-116-treated, respectively) and left ventricular systolic pressure (179 ± 6; untreated, 153 ± 4; delapril-treated, and 158 ± 5 mm Hg; TCV-116-treated, respectively) of sham-operated rats significantly (all $P<0.01$) and to a similar extent. Neither delapril nor TCV-116 changed heart rate (423 ± 13 beats/min, untreated; 419 ± 8 beats/min, delapril-treated; and 434 ± 12 beats/min, TCV-116-treated), left ventricular end-diastolic pressure (3.4 ± 1.2 mm Hg, untreated; 3.8 ± 0.6 mm Hg, delapril-treated; and 1.9 ± 0.6 mm Hg, TCV-116-treated), central venous pressure (2.0 ± 0.4 mm Hg, untreated; 1.7 ± 0.2 mm Hg, delapril-treated; and 2.1 ± 0.2 mm Hg, TCV-116-treated), and left ventricular end-diastolic volume (0.63 ± 0.14 ml/kg body weight, untreated; 0.66 ± 0.10 ml/kg body weight, delapril-treated; and 0.40 ± 0.06 ml/kg body weight, TCV-116-treated) (Fig. 1). Thus, effects on haemodynamic parameters of sham-operated rats did not differ between delapril and TCV-116, although only delapril decreased left ventricular weight.

Comparison of MI rats with sham-operated rats in ventricular weight and haemodynamic parameters

As shown in Table 1, right ventricular weight of untreated MI rats was 1.3-fold ($P<0.01$) larger than that of untreated sham-operated rats. In spite of complete replacement of infarct zone with scarred tissue, left ventricular weight of MI rats was similar to that of untreated sham-operated rats (2.06 ± 0.04 *v* 1.94 ± 0.05 g/kg), thereby showing hypertrophy of the non-infarcted left ventricular wall of MI rats.

As shown by comparison of haemodynamic parameters of untreated sham-operated rats (Fig. 1) with those of untreated MI rats (Fig. 2), left ventricular systolic pressure (152 ± 4 mm Hg, $P<0.01$) in MI rats was lower than sham-operated rats, while left ventricular end-diastolic pressure (13.5 ± 2.0 mm Hg, $P<0.01$), central venous pressure (3.3 ± 0.4 mm Hg, $P<0.05$) and left ventricular end-diastolic volume (2.29 ± 0.15 ml/kg, $P<0.01$) in MI rats were significantly greater compared with those in

TABLE 1. Body weight, infarct size and ventricular weight of sham-operated and MI rats

	Sham-operated rats			MI rats		
	Untreated	Delapril	TCV-116	Untreated	Delapril	TCV-116
n	12	11	10	15	15	21
Body weight (g)	319 ± 8	317 ± 5	304 ± 4	317 ± 5	300 ± 3[1,3]	292 ± 5[2,4]
Infarct size (%)	~	~	~	33.2 ± 1.9	34.0 ± 2.0	33.1 ± 1.5
RV weight (g/kg)	0.57 ± 0.02	0.52 ± 0.02	0.51 ± 0.01[1]	0.74 ± 0.03[2]	0.59 ± 0.01[4]	0.58 ± 0.01[4]
LV weight (g/kg)	1.94 ± 0.05	1.64 ± 0.03[2]	1.85 ± 0.04	2.06 ± 0.04	1.77 ± 0.05[3]	1.86 ± 0.03[4]

Values are mean ± S.E.M., [1]$P<0.05$ and [2]$P<0.01$ compared with untreated sham-operated rats, [3]$P<0.05$ and [4]$P<0.01$ compared with untreated MI rats. RV, right ventricular; LV, left ventricular.

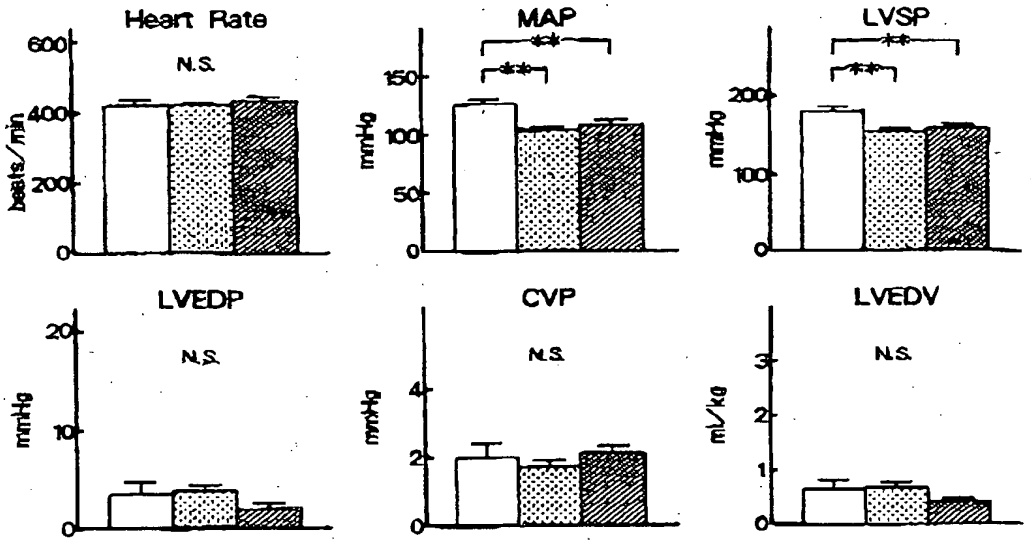

FIGURE 1. Haemodynamic parameters in untreated, delapril-treated and TCV-116-treated sham-operated rats. Bars represent mean ± S.E.M. MAP, mean aortic pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; CVP, central venous pressure; LVEDV, left ventricular end-diastolic volume, $^{**}P < 0.01$, N.S., not significant.

sham-operated rats. On the other hand, there was no difference in heart rate (416 ± 9 beats/min in MI rats) or mean aortic pressure (118 ± 2 mm Hg in MI rats) between the two groups.

Effects of delapril and TCV-116 on ventricular weights and haemodynamic parameters in MI rats

As shown in Table 1, there was no significant difference in infarct size among the three groups of MI rats. Three weeks of treatment

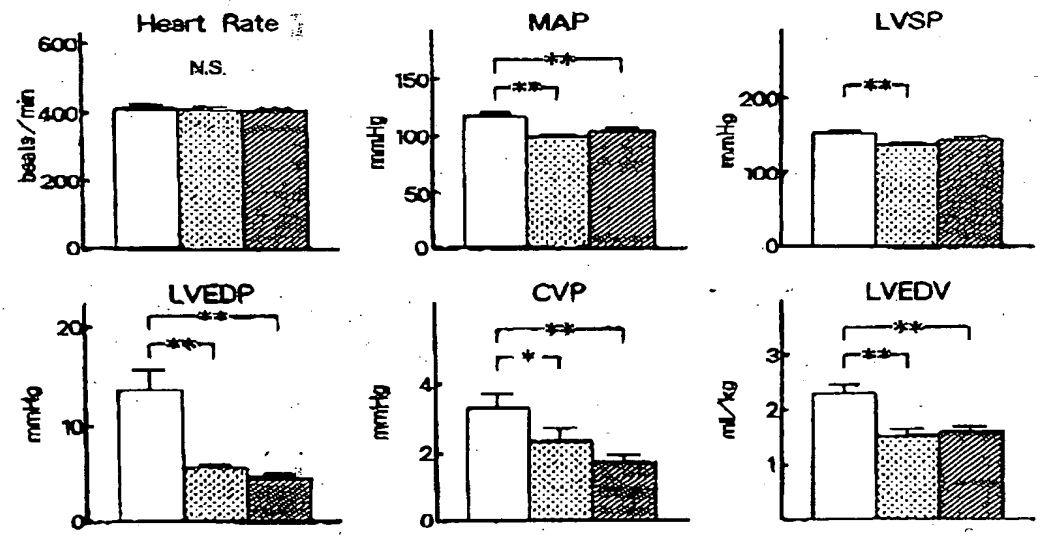

FIGURE 2. Haemodynamic parameters in untreated, delapril-treated and TCV-116-treated MI rats. Bars represent mean ± S.E.M. MAP, mean aortic pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; CVP, central venous pressure; LVEDV, left ventricular end-diastolic volume, $^{*}P < 0.05$, $^{**}P < 0.01$, N.S., not significant.

TABLE 2. Plasma angiotensinogen, renin, Ang II and aldosterone concentrations

	Sham-operated rats: untreated	MI rats		
		Untreated	Delapril	TCV-116
n	12	12	14	11
Infarct size (%)	~	36.3 ± 2.2	36.0 ± 1.3	34.2 ± 1.9
Angiotensinogen (ng Ang I/ml)	1100 ± 93	958 ± 86	203 ± 19[1,2]	508 ± 69[1,2,3]
PRC (ng Ang I/h/ml)	7.8 ± 0.5	13.0 ± 1.5	2554.6 ± 331.4[1,2]	606.4 ± 194.3[3]
Ang II (pg/ml)	6.9 ± 1.5	13.3 ± 3.4	26.2 ± 5.6	57.1 ± 11.4[1,2,3]
Aldosterone (pg/ml)	380 ± 58	360 ± 36	486 ± 36	466 ± 71

Values are mean ± S.E.M., [1]$P < 0.01$ compared with untreated sham-operated rats, [2]$P < 0.01$ compared with untreated MI rats, [3]$P < 0.05$ compared with delapril-treated MI rats, PRC, plasma renin concentration.

with delapril and TCV-116 prevented the increase of right ventricular weight by 20% ($P < 0.01$) and 22% ($P < 0.01$), respectively and reduced left ventricular weight by 14% ($P < 0.01$) and 10% ($P < 0.01$), respectively (Table 1).

Delapril and TCV-116 lowered mean aortic pressure (98 ± 3 and 103 ± 3 mm Hg, respectively), and prevented the increase of left ventricular end-diastolic pressure (5.6 ± 0.3 and 4.6 ± 0.3 mm Hg, respectively), central venous pressure (2.3 ± 0.4 and 1.7 ± 0.2 mm Hg, respectively) and left ventricular end-diastolic volume (1.53 ± 0.10 and 1.59 ± 0.09 ml/kg body weight, respectively), to a similar extent (Fig. 2).

Biochemical studies

Plasma angiotensinogen, renin, Ang II and aldosterone concentrations

Table 2 shows the comparison of the plasma renin-angiotensin system of untreated sham-operated rats, and untreated, delapril-treated and TCV-116-treated MI rats. There was no significant difference in plasma angiotensinogen, renin, Ang II and aldosterone concentrations between untreated sham-operated rats and untreated MI rats.

Delapril and TCV-116 treatments for 3 weeks decreased plasma angiotensinogen by 79% ($P < 0.01$) and 47% ($P < 0.01$), respectively, in MI rats. Plasma renin concentrations in delapril-treated and TCV-116-treated MI rats were approximately 200-fold and 50-fold, respectively, higher than in untreated MI rats. Plasma Ang II concentration in delapril-treated MI rats was slightly higher than that of untreated MI rats, but not to a statistically significant extent. Plasma Ang II in TCV-116-treated rats was 4.3-fold higher ($P < 0.01$) than in untreated MI rats. Plasma aldosterone level was not altered by delapril or TCV-116 treatment of MI rats.

Ang II contents of cardiac ventricular tissues

Figure 3 indicates Ang II contents of right ventricles, non-infarcted left ventricles and

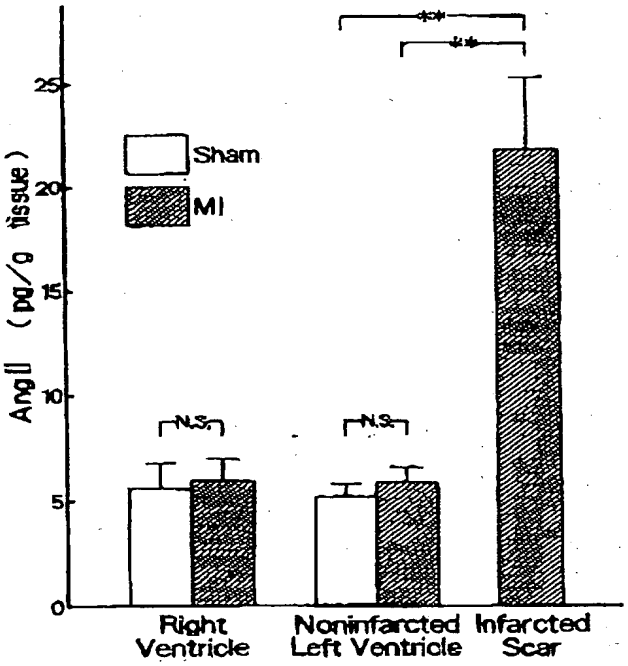

FIGURE 3. Angiotensin II contents of cardiac ventricular tissues from untreated sham-operated rats and untreated MI rats.

Bars represent mean ± S.E.M. **$P < 0.01$. Ang II, angiotensin II.

infarcted scar from untreated sham-operated rats or untreated MI rats. In sham-operated rats, there was no significant difference in Ang II contents between the right and left ventricles (5.6 ± 1.2 *v* 5.2 ± 0.6 pg/g tissue). Ang II contents of the right ventricle (6.0 ± 1.0 pg/g tissue) and the non-infarcted left ventricle (5.9 ± 0.7 pg/g tissue) in MI rats were not different from those in sham-operated rats. Ang II content of the scarred tissue of MI rats (21.7 ± 3.5 pg/g tissue) was 4.2-fold ($P<0.01$) and 3.7-fold ($P<0.01$) higher than that of the left ventricle of sham-operated rats and of the non-infarcted left ventricle of MI rats, respectively.

As indicated by Figure 4, delapril treatment of MI rats for 3 weeks decreased Ang II contents of the right and non-infarcted left ventricles, and the scarred tissue by 48% ($P<0.05$), 81% ($P<0.01$) and 60% ($P<0.01$), respectively. TCV-116 also decreased Ang II contents of the right and non-infarcted left ventricles by 57% ($P<0.01$) and 56% ($P<0.01$), respectively, but did not significantly change that of the scarred tissue.

Discussion

ACE inhibitors and AT_1 receptor antagonist have been widely accepted to have favourable effects on ventricular dilatation and heart failure after myocardial infarction (Gay, 1990; Lamas and Pfeffer, 1991; Pfeffer *et al.*, 1985; Raya *et al.*, 1991; Sharpe *et al.*, 1991). Therefore, renin-angiotensin system is thought to play an important role in pathophysiology of ventricular dilatation and heart failure. In this study, chronic ACE inhibition with delapril reduced mean aortic pressure and left ventricular systolic pressure, and prevented the increase of left ventricular end-diastolic pressure, central venous pressure, and right and left ventricular weights completely, and the increase of left ventricular end-diastolic volume partially in MI rats (Table 1 and Fig. 2). These observations were in good agreement with the previous findings obtained for captopril (Gay, 1990). AT_1 receptor-blockade with TCV-116 in MI rats led to similar haemodynamic effects to delapril, with respect to mean aortic pressure, left ventricular end-diastolic pressure, central

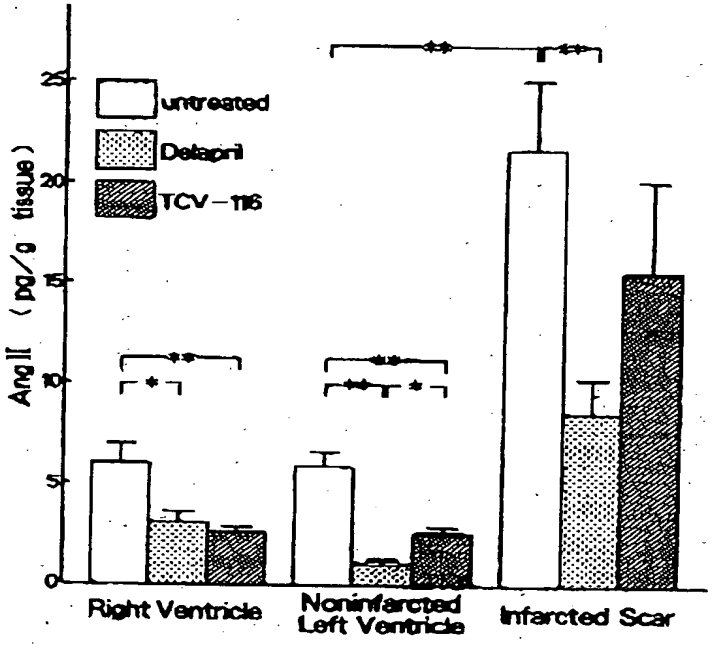

FIGURE 4. Angiotensin II contents of cardiac ventricular tissues from untreated, delapril-treated and TCV-116-treated MI rats.

Bars represent mean ± S.E.M. $*P<0.05$, $**P<0.01$. Ang II, angiotensin II.

venous pressure and left ventricular end-diastolic volume. These results reveal that both delapril and TCV-116 are effective in partial prevention of ventricular dilatation after myocardial infarction.

Raya *et al.* (1991) investigated the effect of 2 weeks of treatment with the non-peptide AT_1 receptor antagonist, Dup 753 (40 mg/kg/day) in the rat model of heart failure at 3 weeks after coronary ligation. In their study, Dup 753 prevented the increase of left ventricular end-diastolic pressure and left ventricular end-diastolic volume. However, Dup 753 failed to prevent the increase of right atrial pressure, or right and left ventricular weight in rats with heart failure, findings different from our present observations for TCV-116. We have found that the oral doses needed for 50% inhibition of pressor response to Ang II (100 ng/kg, i.v.) in rats were 0.06 mg/kg and 2.89 mg/kg for TCV-116 and Dup 753, respectively, thereby indicating that TCV-116 has about 50-fold stronger potency than Dup 753. Thus, the discrepancy between the results obtained by us and Raya *et al.* (1991), may be explained by the difference in the *in vivo* potency between the two AT_1 receptor antagonists. However, while they investigated the effect of 2 weeks of treatment with Dup 753 in the rats at 3 weeks after coronary ligation, we investigated the effect of 3 weeks of treatment with TCV-116 in the rats immediately after coronary ligation. Therefore, it cannot be excluded that the discrepancy might be due to the differences in the experimental designs, including the time of start of the drug treatment and the period of the treatment.

Accumulating evidence indicates that in addition to the circulating renin-angiotensin system, the local renin-angiotensin system exists in the heart (Baker *et al.*, 1984; Dzau, 1987; Lindpainter and Ganten, 1991). The demonstration of mRNA for angiotensinogen, renin and ACE indicates that all the components can be synthesized within cardiac tissue, and the existence of receptor of Ang II in ventricle (Baker *et al.*, 1984) indicates that this system functions in ventricle. However, the regulating mechanism for the generation of Ang II within the heart is still unknown. Recent *in vitro* studies, using human cardiac tissue, indicate that cardiac Ang II can be produced to a significant amount by the ACE-independent pathway (Urata *et al.*, 1990a,b), namely the alternative pathway. Therefore, the direct measurement of Ang II in cardiac tissues is essential for the understanding of the pathophysiological role of cardiac renin-angiotensin system.

In the present study, we examined cardiac Ang II contents in MI rats and the effects of the ACE inhibitor and the AT_1 receptor antagonist on cardiac Ang II contents. Anti-Ang II serum, used in the present study, cross-reacted with Ang I only by 0.2%, but with the related fragments of Ang II, including Ang III, Ang (3–8) and Ang (4–8), by 100%, thereby indicating that our polyclonal antibodies recognize the carboxyl-terminal portion of Ang II peptide. However, reverse phase HPLC separation, prior to RIA of Ang II, allowed for the complete separation of true Ang II from the other related peptides. Thus, true Ang II in cardiac ventricular tissues and plasma could be measured in the present study.

Right and left ventricular Ang II contents in sham-operated and MI rats were comparable to those in plasma, being consistent with the data obtained for normal rats (De Silvia *et al.*, 1988). The values of cardiac Ang II contents measured in the present study represent intracardiac Ang II rather than Ang II in the contaminated blood, because the contaminated blood was exhaustively washed out from the heart by saline infusion after decapitation of rats.

Of note are the observations that Ang II contents were significantly increased in infarcted scar tissue, but not in the right or non-infarcted left ventricles of untreated MI rats (Fig. 3). The present work cannot explain the exact reason for the increased Ang II content in the infarcted scar of MI rats. However, this may be explained by the findings by Johnston *et al.* (1991), who found marked increase of ACE in the infarcted scar tissue, using autoradiographic techniques. The myocardium in infarct zone is completely replaced with fibroblasts accompanied by increased deposition of collagen at 3 weeks after coronary ligation (Fishbein *et al.*, 1978). Recent investigation on effects of chronic infusion of Ang II in rats indicate that Ang II causes fibroblast proliferation and scar formation in heart (Tan *et al.*, 1991). These findings

suggest that the enhanced production of Ang II in the scarred tissue may participate in proliferation of fibrous tissue in infarct zone by autocrine or paracrine mechanism of Ang II, resulting in progression of infarct expression.

Hirsch *et al.* (1991) report that the expression of ACE mRNA and its enzyme activity are increased in the non-infarcted myocardium at 12 weeks after coronary ligation of rats. Therefore, they speculated that local production of Ang II in the non-infarcted and hypertrophied myocardium in MI rats is accelerated. Interestingly, in the present investigation, Ang II content was increased in neither right nor non-infarcted left ventricles in MI rats, in spite of obvious ventricular dilatation and hypertrophy. The dissociation of the Ang II level with the level of other components of the renin-angiotensin system has also been reported in adrenal gland by Kim *et al.* (1992), who have found the dissociation of Ang II with renin in the adrenal gland of rats. Local tissue content of the peptide depends on the balance of production, receptor-mediated internalization and degradation. Thus, it is possible that in non-infarcted and hypertrophied ventricles of MI rats, the rate of receptor-mediated internalization or degradation of Ang II as well as ACE-mediated Ang II synthesis might be increased, resulting in no elevation of Ang II content.

Three weeks of treatment with delapril significantly reduced Ang II contents of the right and non-infarcted left ventricles, and the infarcted scar. On the other hand, plasma Ang II level tended to increase by chronic ACE inhibition, a finding consistent with the previous reports (Campbell *et al.*, 1991; Mento and Wilkes, 1987). Chronic ACE inhibition increased the plasma renin activity by feedback mechanism. Markedly increased plasma renin would activate to convert angiotensinogen to angiotensin I, resulting in decrease of angiotensinogen and marked increase of plasma Ang I. The markedly increased Ang I may be converted to Ang II by ACE, which is not inhibited completely by ACE inhibitor. These results demonstrate that effects of chronic ACE inhibitor treatment on ventricular dilatation is mainly caused by the inhibition of local Ang II production in myocardial tissue rather than in the circulation, and that in ventricles of MI rats ACE-mediated pathway rather than the alternative pathway plays a major role in cardiac Ang II synthesis. Furthermore, the dissociation between Ang II levels of plasma and ventricular tissues provides evidence for tissue-specific regulation of cardiac renin-angiotensin system, as in the case of kidney (Campbell *et al.*, 1991) and adrenal gland (Kim *et al.*, 1992) of rats.

AT_1 receptor antagonism increased plasma renin activity by a feedback mechanism. This would result in decrease of angiotensinogen and increase of Ang II in plasma by acceleration of conversion of angiotensinogen to Ang I. In the present study, we found that AT_1 receptor blockade reduced Ang II contents of the right and non-infarcted left ventricles, in contrast to the increase in the plasma Ang II level. This provides further evidence for independent and tissue-specific regulation of the cardiac renin-angiotensin system. Moreover, this finding may indicate that the internalization of Ang II via the cardiac AT_1 receptor plays an important role in ventricular Ang II level. On the contrary, TCV-116 did not markedly decrease the Ang II content of scarred tissue. Therefore, there may be some difference in the property of the Ang II receptor between the non-infarcted ventricles and the infarcted scar tissue.

The response of renin-angiotensin system after myocardial infarction is time-dependent (Drexler *et al.*, 1989). However, in the present study, we investigated the renin-angiotensin system on a single time of 3 weeks after myocardial infarction. For further understanding of the contribution of the renin-angiotensin system to ventricular dilatation after myocardial infarction, the time course of response of the renin-angiotensin system after myocardial infarction has to be investigated.

In conclusion, ACE inhibition and AT_1 receptor blockade prevented the ventricular dilatation after myocardial infarction, which was associated with the decrease in ventricular Ang II contents, not with the decrease of the plasma Ang II level. These results, taken together with the observation on the increased Ang II level in the infarcted scar tissue of ventricle, suggest that the cardiac renin-angiotensin system rather than the circulating system may play an important role in the

development of ventricular dilatation after myocardial infarction.

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